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RELATION OF PANTOTHENIC ACID TO THE FILTRATE FRACTION OF THE VITAMIN B COMPLEX

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(Received for publication July 20, 1940)

The occurrence of an experimentally produced nutritional dermatitis in chickens was first described by Ringrose, Norris and Heuser ('31). Kline, Keenan, Elvehjem and Hart ('32) introduced the heated diet upon which dermatitis could be produced quite regularly. Elvehjem and Koehn ('35) showed this diet to be deficient in a "filtrate factor," while Lepkovsky and Jukes ('35) reported the heated diet to be also low in riboflavin. The latter investigators supplemented the diet with a fuller's earth adsorbate of riboflavin prepared from whey, thereby improving the heated diet as a test for their "filtrate factor." Bauernfeind and Norris ('39) subsequently found the heated diet to be deficient in a new growth factor which they showed could be adsorbed on fuller's earth.

The heated diet has been widely used to evaluate quantitatively the amount of chick antidermatitis vitamin ("filtrate factor") in foodstuffs. Waisman, Mickelsen and Elvehjem ('39) determined the minimum amount of foodstuff necessary to prevent dermatitis and made their comparisons on this basis. Jukes and Lepkovsky ('36) used growth over a 14-day period as the basis for their "filtrate factor unit." The use of growth measurements for quantitative comparisons would be valid only if there were no other growth limiting deficiency in the diet, but the findings of Bauernfeind and Norris ('39) indicate the heated diet may not meet this requirement.

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The chick antidermatitis vitamin has been shown by Woolley, Waisman and Elvehjem ('39) and Jukes ('39) to be similar to pantothenic acid. Filtrates containing the chick antidermatitis vitamin also contain a factor or factors necessary for the rat, and such filtrates have been studied with rats to secure information on this factor (factor 2 or "rat filtrate factor"). Hitchings and Subbarow ('39) on the basis of stability data and Macrae, Todd, Lythgoe, Work, Hind and El Sadr ('39) on the basis of solubility have claimed that the "filtrate factor" consists of two or more growth-promoting vitamins. On the basis of solubility in ether from strongly acidified solutions, Mohammad, Emerson, Emerson and Evans ('40) and Kringstad and Lunde ('39) have concluded that the chick antidermatitis vitamin is different from the "rat filtrate factor."

Very potent concentrates of pantothenic acid were shown by Oleson, Woolley and Elvehjem ('39) and Subbarow and Hitchings ('39) to stimulate growth in rats. Woolley ('40) confirmed this with pure pantothenic acid and was able to show definitely that rats require an unidentified factor of the vitamin B complex or an unidentified water-soluble factor in addition to pantothenic acid, thiamin, riboflavin and pyridoxin.

Lunde and Kringstad ('39) and Morgan and Simms ('40) have reported the occurrence of an "anti-grey hair factor" in "filtrates" which appears to be different from the known vitamins. Gyorgy, Poling and Subbarow ('40) have found pantothenic acid to play an important role in the cure of grey hair. Their pantothenic acid concentrates were about 50% pure. Oleson, Elvehjem and Hart ('39) have shown that while grey hair is not prevented with pantothenic acid, it does definitely delay the onset of the symptoms.

Nielsen, Oleson and Elvehjem ('40) have described the procedure for the preparation of a crystalline fraction from liver, which is not pantothenic acid, but which seems to be specifically active in the prevention of the greying of black rats' hair (nutritional achromotrichia). It is possible that nutritional achromotrichia is influenced by several factors.

The synthesis of pantothenic acid by Williams and Major ('40) and Weinstock, Arnold, May and Price ('40) has made possible a further study of the "filtrate" factors of the vitamin B complex for both chickens and rats.

EXPERIMENTAL

Chicks. Day-old chicks were placed on the U. C. Poultry Mash (Lepkovsky and Jukes, '35) for 1 week and maintained on the heated diet for 1 week at which time the tests were started. The composition of the heated diet is as follows:

	%	
Whole ground corn	55.25	Heated at 120°C. for 24 hours
Wheat middlings	25.00	
Extracted casein [1]	12.00	
B-80 adsorbate [2]	2.50	
Sodium chloride	1.00	
Ground limestone	1.00	
Steamed bonemeal	1.00	
Cod liver oil	2.00	
Alfalfa leaf meal	0.25	

All chicks were allowed the diet and water ad libitum.

The test materials were diluted to a convenient volume for feeding. They were fed by inserting a pipette into the esophagus of the chick and allowing the material to flow into the crop. The doses were fed daily with a double dose on Saturday to take care of the Sunday feeding.

Development and disappearance of typical chick dermatitis were noted throughout the experimental period.

Rats. Twenty-one-day-old rats weighing 40 to 50 gm. were placed in individual cages with wire screen bottoms and fed the basal diet (Dimick and Schreffler, '39). The diet consisted in per cent of sucrose 59, extracted casein 27, primex 8, cod liver oil 2, and salt mixture no. 185 (McCollum and Simmonds, '18) 4. It was supplemented daily with 25μg. each of thiamin, riboflavin and pyridoxin.

[1] As described by Dimick and Schreffler ('39).

[2] B-80 adsorbate prepared from rice bran concentrate was assayed separately and contained 80 U.S.P. units of thiamin, 60 μg. of riboflavin and 50 μg. of pyridoxin per gram.

The rats were weighed weekly and when they failed to gain, they were started on the test materials. This occurred in from 4 to 5 weeks when the body weight was from 55 to 75 gm. The fur was ruffled, greasy in appearance and showed indications of greying. Some rats had developed reddening and swelling of the eyelids with an exudate which frequently caused the lids to stick together.

Dietary supplements. A rice bran concentrate [3] was used as the source of the vitamin B complex. Microbiological tests [4] as used by Weinstock et al. ('40) showed this concentrate to contain 400 μg. of pantothenic acid per cubic centimeter. A microbiologically assayed crude preparation of synthetic pantothenic acid served as our source of this factor.[5]

The eluate was prepared as follows: 500 gm. fuller's earth were shaken with 1 liter of rice bran concentrate diluted with 5 volumes of water at pH 5.5. This adsorbate was washed thoroughly. It was eluted with 0.1 N barium hydroxide solution. After removal of the barium the remaining liquid was concentrated under vacuum.

The rice bran filtrate fraction was prepared as follows: The rice bran concentrate was treated twice with fuller's earth using 500 gm. fuller's earth for each 1 liter of rice bran concentrate diluted to 5 volumes with water. The filtrate was concentrated and fermented with yeast to remove the sugar. It was adjusted to pH 7 with calcium hydroxide, concentrated, and methanol added to a concentration of about 80% by volume. It was then filtered and concentrated. This concentrate was adjusted to pH 2 and extracted in the cold six times with 80% methyl ethyl ketone. The methyl ethyl ketone insoluble fraction, after removal of the ketone, was treated three times with charcoal at pH 4–5. Two hundred and fifty

[3] Essentially an aqueous extract of rice bran commercially known as "Vitab Type II Liquid."

[4] We are indebted to Dr. Aaron Arnold, National Oil Products Company, for the microbiological tests of rice bran concentrate (Vitab Type II Liquid), pantothenic acid, and rice bran filtrate fraction.

[5] This was supplied by Doctors Weinstock, Arnold, May and Price, National Oil Products Company.

grams of charcoal and 1 liter of ketone insoluble fraction, diluted with 5 volumes of water, were used for each adsorption. The final filtrate, concentrated to a thick syrup, contained only 14 μg. of pantothenic acid per cubic centimeter by microbiological test.[6]

Feeding experiments. Groups of chicks were fed the rice bran concentrate at levels of 1.0, 0.5, 0.25 and 0.125 cc. respectively per chick per day. The pantothenic acid at each level was calculated and other groups of chicks were fed equivalent levels of the synthetic pantothenic acid. The differences in growth between chicks on the rice bran concentrate and those on pantothenic acid were noted, and, since these differences might well be due to the factor of Bauernfeind and Norris ('39) this factor was fed as a barium hydroxide eluate of fuller's earth. One-tenth cubic centimeter of the eluate per chick per day was given to half of each group of chicks which had previously received 400 and 200 μg. of pantothenic acid respectively for the 3 weeks' test period.

Rats were similarly studied. The rice bran concentrate containing 400 μg. pantothenic acid per cubic centimeter was fed at levels of 1.0 and 0.5 cc. respectively. Synthetic pantothenic acid was fed at levels of 800, 400 and 200 μg. respectively. Since the rice bran concentrate promoted greater growth in the rats than synthetic pantothenic acid it seemed logical to assume that the concentrate was furnishing an unidentified water-soluble factor or factors of the vitamin B complex. Accordingly, the rice filtrate fraction containing negligible amounts of pantothenic acid was fed in addition to pantothenic acid. Three groups of rats received 50, 100 and 200 μg. pantothenic acid respectively, and three groups received the corresponding amounts of pantothenic acid plus 0.5 cc. rice bran filtrate fraction. This level supplied 7 μg. of pantothenic acid which amount was subtracted in feeding the pantothenic acid doses.

[6] See footnote 4, page 416.

RESULTS

Chicks. The average growth rates of chicks fed pantothenic acid was inferior to that of chicks fed the same amount of pantothenic acid in the form of rice bran concentrate. This is shown in table 1.

TABLE 1

Comparative growth of chicks receiving rice bran concentrate and equivalent levels of pantothenic acid

SUPPLEMENT	NUMBER OF CHICKS	AVERAGE BODY WEIGHT AT				DERMATITIS
		Beginning	1 week	2 weeks	3 weeks	Gain	
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	
Rice bran concentrate 1.0 cc.: equivalent to 400 μg. pantothenic acid	8	83	127	190	237	154	None
400 μg. pantothenic acid	8	82	122	166	210	128	None
Rice bran concentrate 0.5 cc.: equivalent to 200 μg. pantothenic acid	8	83	116	186	230	147	None
200 μg. pantothenic acid	8	84	115	171	203	119	None
Rice bran concentrate 0.25 cc.: equivalent to 100 μg. pantothenic acid	8	83	109	152	190	107	4 mild cases
100 μg. pantothenic acid	8	83	106	125	161	78	6 severe cases
Rice bran concentrate 0.125 cc.: equivalent to 50 μg. pantothenic acid	8	83	100	134	149	66	2 died 3 severe 2 mild
50 μg. pantothenic acid	8	83	92	127	144	61	3 died 4 severe 1 mild

The chicks receiving 1.0 and 0.5 cc. levels of rice bran concentrate respectively, as well as those receiving 400 and 200 μg. levels of pantothenic acid respectively, were completely free of dermatitis. Those receiving the next lower levels of rice bran concentrate and pantothenic acid showed incomplete protection, while the lowest levels afforded the chicks only slight protection. The protection against chick dermatitis seemed consistent with the level of pantothenic acid fed whether from rice bran concentrate or as the synthetic preparation.

The difference in growth between chicks receiving the rice bran concentrate and those given synthetic pantothenic acid

TABLE 2

Growth of chicks receiving pantothenic acid with and without rice bran eluate

SUPPLEMENT	NUMBER OF CHICKS	AVERAGE BODY WEIGHT AT			Gain
		Beginning	1 week	2 weeks	
		gm.	*gm.*	*gm.*	*gm.*
200 μg. pantothenic acid	4	217	249	286	69
200 μg. pantothenic acid plus 0.1 cc. eluate	4	202	255	328	116
400 μg. pantothenic acid	4	213	269	308	95
400 μg. pantothenic acid plus 0.1 cc. eluate	4	208	271	338	130

indicated that another factor is necessary for the chick fed the heated diet supplemented with pantothenic acid. The lack of this factor was not evident until after the end of the first week of the test period, but became striking by the end of 3 weeks. On this basis, it would seem that dermatitis, used by Waisman, Mickelsen and Elvehjem ('39), may be a better criterion than growth, used by Jukes and Lepkovsky ('36), in the quantitative assay of foodstuffs for pantothenic acid.

The results of feeding an eluate in addition to 400 and 200 μg. pantothenic acid are shown in table 2. The eluate increased the growth in both cases, showing that it must contain a factor other than pantothenic acid. As the factor can be

adsorbed on fuller's earth, it may be similar to that of Bauernfeind and Norris ('39), who have shown that the mature fowl, when fed the heated diet supplemented with pantothenic acid, supplied by rice bran filtrate, also requires an adsorbable factor. Our factor may also be related to factor U of Stokstad and Manning ('38) which has similar properties.

Rats. The results with rats are shown in tables 3 and 4. The rice bran concentrate supported a better rate of growth than synthetic pantothenic acid (table 3), even when four times as much pantothenic acid as that supplied by the rice

TABLE 3

Comparative growth of rats receiving rice bran concentrate and panthothenic acid

SUPPLEMENT	NUMBER OF RATS	SEX	AVERAGE BODY WEIGHT AT Beginning	7 weeks	Gain
			gm.	gm.	gm.
Rice bran concentrate 0.5 cc.: equivalent to 200 μg. pantothenic acid	6	Females	60	191	131
200 μg. pantothenic acid	6	Females	67	172	105
Rice bran concentrate 1.0 cc.: equivalent to 400 μg. pantothenic acid	4	2 males 2 females	71	233	162
400 μg. pantothenic acid	3	2 males 1 female	72	154	82
800 μg. pantothenic acid	3	2 males 1 female	99	196	97

bran concentrate was fed. Pantothenic acid cured the eye symptoms, improved the fur condition and stimulated rapid growth. The growth of rats receiving synthetic pantothenic acid, however, was not as marked as that of rats receiving equivalent amounts of pantothenic acid present in the rice bran concentrate. Rats which were grey on the back, as well as one which had the fur missing in patches on the sides with slight skin eruptions exhibited growth of fur of a normal color, and the skin eruptions cleared up when pantothenic acid was fed. However, the new fur was subsequently inclined to

turn grey and considerable greying persisted. Twelve rats (used for other experiments) were fed 200 μg. daily of synthetic pantothenic acid from the day of weaning in addition to the same basal diet and 25 μg. each of thiamin, riboflavin, and pyridoxin. At 3 months of age, nine of the twelve showed

TABLE 4

Comparative effect of feeding various levels of pantothenic acid with and without the rice bran filtrate fraction with adjustment for the pantothenic acid contained in the filtrate fraction

SUPPLEMENT	NUMBER OF RATS	AVERAGE BODY WEIGHT AT Beginning	7 weeks	Gain	FUR CONDITION
	Females	*gm.*	*gm.*	*gm.*	
50 μg. pantothenic acid	4	58	111	53	Grey
43 μg. pantothenic acid plus 0.5 cc. filtrate fraction containing 7 μg. pantothenic acid	5	59	150	91	3 grey 2 restored
100 μg. pantothenic acid	4	57	147	90	grey
93 μg. pantothenic acid plus 0.5 cc. filtrate fraction containing 7 μg. pantothenic acid	4	57	167	110	3 restored 1 some grey remaining
200 μg. pantothenic acid	5	63	150	87	Some grey
193 μg. pantothenic acid plus 0.5 cc. filtrate fraction containing 7 μg. pantothenic acid	4	59	177	118	None grey

slight signs of greying. This indicates that pantothenic acid does not completely prevent greying.

The rats receiving the rice bran concentrate were superior in that respect. This is in agreement with previous unpublished experiments with rice bran concentrates which have been shown to contain a factor or factors which will prevent greying and restore the normal fur color in rats which have become grey.

This is in agreement with the published results of Morgan and Simms ('40), which have shown rice bran concentrate to be an excellent source of the "anti-grey hair factor."

The data in table 4 point definitely to an unidentified "filtrate factor" which escapes, at least in part, adsorption by fuller's earth and charcoal. Rats receiving the rice bran filtrate fraction in addition to pantothenic acid grew better at all levels than those on the corresponding levels of pantothenic acid. The hair color of rats receiving 200 μg. pantothenic acid plus the filtrate fraction was completely restored in the 7 weeks' test period. The hair of the rats receiving the 100 μg. pantothenic acid plus the filtrate fraction was definitely black with the greying disappearing rapidly. This was in contrast to those on pantothenic acid alone and similar to those on the rice bran concentrate. When fed alone, the rice bran filtrate fraction possessed no demonstrable biological activity.

DISCUSSION

It is clear that the vitamin B complex consists of still unidentified additional factors. Evidence has been presented to show that at least two unidentified factors belonging to the vitamin B complex are present in a concentrate prepared from rice bran. These factors would appear to be different since one, needed by the chick, is adsorbable on fuller's earth; and the other, needed by the rat, is found in filtrates after treatment with fuller's earth and charcoal. Bauernfeind and Norris ('39) showed adsorbates to be effective in the chick in the presence of rice bran filtrate.

Pantothenic acid is an important fraction of the rat "filtrate factor" or factor 2, but does not constitute all of it by any means. It supports rapid growth, but at a rate lower than that obtained when a rice bran concentrate is fed. Pantothenic acid clears up such minor factor 2 deficiency symptoms as swollen eyelids and occasional skin eruptions. The rats have a slightly greasy appearance with ruffled fur, and grey hairs sprinkled throughout, but are otherwise not very different from normal animals.

Pantothenic acid seems to be an important component of the "anti-grey hair factor," delaying greying and causing darkening of the fur already grey. Nielsen, Oleson and Elvehjem ('40) have prevented greying with a crystalline preparation from liver. The relation between this factor and pantothenic acid in the prevention of greying is, at present, not clear. Some vitamins such as pyridoxin and nicotinic acid (Morgan and Simms, '40; Lunde and Kringstad, '40) accelerate the greying of the rats' fur. It would seem that greying is a phenomenon involving many factors whose interrelationships are at present not well understood.

Our results show that, in the chicken, pantothenic acid is definitely identical with the chick antidermatitis vitamin, but only insofar as prevention and cure of dermatitis is concerned. It does not comprise all of the filtrate factor, since the filtrate factor is measured in terms of growth on the heated diet and since it has been demonstrated that there is at least one unidentified growth factor other than pantothenic acid which is inadequately supplied by the heated diet.

The heated diet is satisfactory for the quantitative estimation of pantothenic acid when the prevention of dermatitis is the criterion used (Waisman, Mickelsen and Elvehjem, '39). If growth is the criterion, however, only those foodstuffs free of unidentified growth promoting factors will give reliable results.

CONCLUSIONS

1. Synthetic pantothenic acid will cure or prevent an experimentally produced nutritional dermatitis which develops in chicks on a diet of natural foodstuffs heated at 120°C. for 24 hours. It does not appear to represent the whole of the factor referred to as the "filtrate factor" since it produces a growth response below that obtained with rice bran concentrate.

2. An eluate prepared from rice bran concentrate contains a factor necessary for the growth of chicks on the heated diet

of natural foodstuffs in addition to pantothenic acid. This confirms the findings of Bauernfeind and Norris ('39).

3. Pantothenic acid is an important part of factor 2 ("rat filtrate factor"). It promotes growth of rats, cures the reddening and swelling of the eyelids which develop in rats on a factor 2-deficient diet, improves the skin condition and decreases greying of the fur; but it does not completely restore hair color nor does it completely prevent greying.

4. From a rice bran concentrate, a filtrate fraction low in pantothenic acid has been prepared which is shown to contain an unidentified factor necessary for the rat.

5. Rice bran concentrate contains a chick and a rat growth factor (or factors) in addition to the now recognized components of the vitamin B complex, namely, thiamin, riboflavin, pyridoxin, nicotinic acid and pantothenic acid.

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VITAMIN C METABOLISM IN EASTERN COTTON RATS [1]

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Recent demonstration of the susceptibility of the eastern cotton rat to the virus of poliomyelitis (Armstrong, '39; Jungeblut and Sanders, '40) prompted a systematic investigation in this laboratory of the response of this animal to other infectious and toxic agents. The significant finding which emerged from these comparative studies was the fact that the cotton rat in its reaction to diphtheria toxin (Jungeblut, '40), histamine poisoning (Seegal, '40) and experimental tuberculosis (Steinbach and Duca, '40) followed a rather uniform pattern. To be more precise, its tolerance to these agents seemed to correspond with that of an animal occupying a position, within the rodent group, midway between the extremely susceptible guinea pig, on the one hand, and the extremely insusceptible albino rat, on the other. Admittedly, the mechanism involved in the various manifestations of natural tolerance is still far from being well understood; however, there are reasons for believing that basic differences in vitamin C metabolism may play an important, if not decisive part, at least in the above-mentioned instances (Perla and Marmorston, '37).

In view of the described peculiar biological grouping of the cotton rat it has seemed of interest to determine whether this animal, with respect to its vitamin C metabolism, should

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be regarded as belonging to the non-synthesizing type of the guinea pig, or to the synthesizing type of the albino rat. Ascorbic acid determinations were therefore carried out in which the vitamin C levels in the tissues of cotton rats were compared with those of guinea pigs and albino rats. In further experiments, the effect of withdrawal of vitamin C from the diet upon the vitamin C content of representative organs was studied in the same three species.

Florida cotton rats (Sigmodon hispidus littoralis), weighing between 100 and 175 gm., were used in this work. Before being placed on an experiment, the animals had been kept in the laboratory for at least 30 days on an adequate diet (oatmeal, raisins, apples and greens). The albino rats were laboratory-bred (weight from 100 to 150 gm.) and had been maintained on a modified McCollum diet. The guinea pigs (weight from 250 to 300 gm.) had been on an adequate laboratory diet (oats, hay and greens) for short periods of time, ranging from 4 to 10 days. All animals were sacrificed and the tests made on fresh tissues. The organs examined for vitamin C content were liver, brain, spleen and adrenal. The modified method of Bessey and King ('33) and Musulin and King ('36) was employed for determining the amounts of reduced ascorbic acid present in these tissues.

Vitamin C content of tissues of cotton rats, albino rats and guinea pigs

During the spring and early summer months, a total of eight cotton rats were killed and ascorbic acid titrations made on representative tissues. On the same days, for purpose of comparison, determinations were also made on the tissues of albino rats and guinea pigs. The results are given in table 1.

It will be seen that the average figures for the vitamin C content of liver, brain and spleen show no significant differences between cotton rats and albino rats. However, the vitamin C levels in the adrenals of cotton rats were definitely lower than those in the adrenals of albino rats. Thus, the

TABLE 1

Vitamin C content of the tissues of cotton rats and effect of scorbutic diet on its level as compared with the effect on the level in tissues of albino rats and guinea pigs

ANIMAL	NUMBER	DIET	NUMBER OF DAYS ON DIET	MILLIGRAMS OF ASCORBIC ACID PER GRAM OF FRESH TISSUE			
				Liver	Brain	Spleen	Adrenals
Guinea pig	10 (average)	Stock	4–10	0.075	0.085	0.082	0.29
Guinea pig	2	Scorbutic + ascorbic acid	15	0.13	0.14	0.12	0.501
Guinea pig	1	Scorbutic + ascorbic acid	22	0.089	0.15	0.19	0.38
Guinea pig	1	Scorbutic + ascorbic acid	30	0.11	0.12	0.23	0.36
Guinea pig	1	Scorbutic + ascorbic acid	31	0.15	0.15	0.21	0.55
Guinea pig	2	Scorbutic	15	0.018	0.017	0.019	0.036
Guinea pig	1	Scorbutic	22	0.035	0.017	0.02	0.044
Cotton rat	8 (average)	Stock	+30	0.22	0.28	0.34	1.13
Cotton rat	2	Scorbutic + ascorbic acid	17	0.37	0.32	0.37	1.14
Cotton rat	1	Scorbutic + ascorbic acid	25	0.22	0.30	0.58	1.10
Cotton rat	1	Scorbutic + ascorbic acid	37	0.25	0.29	0.50	1.09
Cotton rat	1	Scorbutic + ascorbic acid	51	0.28	0.33	0.46	1.32
Cotton rat	2	Scorbutic	17	0.31	0.29	0.44	0.934
Cotton rat	1	Scorbutic	25	0.22	0.32	0.40	0.99
Cotton rat	1	Scorbutic	37	0.27	0.28	0.47	0.85
Cotton rat	1	Scorbutic	51	0.29	0.30	0.54	1.27
Albino rats	16 (average)	Stock	±60	0.23	0.32	0.28	2.59
Albino rat	2	Scorbutic + ascorbic acid	18	0.18	0.31	0.23	2.70
Albino rat	1	Scorbutic + ascorbic acid	25	0.15	0.35	0.21	3.21
Albino rat	1	Scorbutic + ascorbic acid	39	0.21	0.31	0.25	3.46
Albino rat	1	Scorbutic + ascorbic acid	50	0.25	0.33	0.34	2.95
Albino rat	1	Scorbutic + ascorbic acid	51	0.24	0.31	0.29	3.05
Albino rat	2	Scorbutic	18	0.11	0.20	0.15	2.67
Albino rat	1	Scorbutic	25	0.21	0.34	0.23	3.32
Albino rat	1	Scorbutic	39	0.20	0.31	0.28	3.33
Albino rat	1	Scorbutic	50	0.20	0.31	0.29	2.93
Albino rat	1	Scorbutic	51	0.17	0.32	0.30	3.39

average vitamin C content, expressed in milligrams of ascorbic acid per gram of fresh tissue, was 2.59 in the adrenals of the albino rat (range 2.00 to 3.34), whereas in the cotton rat it was only 1.13 (range 1.10 to 1.15). On the other hand, the average vitamin C levels of all cotton rat tissues were considerably higher than those of the corresponding tissues for guinea pigs. Although the average figures for guinea pigs given in this table are clearly below normal, probably due to a combination of inadequate diet and seasonal changes (Jungeblut and Feiner, '39), yet the values obtained for the tissues of other guinea pigs, known to have received ample amounts of ascorbic acid, remained consistently below those of cotton rat tissues.

The effect of withdrawal of vitamin C from the diet on the vitamin C content of the tissues of cotton rats, albino rats and guinea pigs

A total of twelve cotton rats, twelve albino rats and twelve guinea pigs were included in this experiment. After having been kept on a preliminary stock diet for 7 days, the members of each species were distributed into two groups of six animals each. One group was fed a modified Sherman scorbutic diet, consisting of rolled oats, skimmed milk powder, cottonseed oil,[2] cod liver oil and sodium chloride, with wheat bran for the guinea pigs and ground whole wheat for the rats. The other group was maintained on the same scorbutic diet but received in addition large doses of synthetic ascorbic acid [3] dissolved in fresh orange juice. Each animal ingested daily approximately from 5 to 10 mg., an amount many times the prophylactic antiscorbutic dose for guinea pigs. At intervals, animals were killed and ascorbic acid determinations made on representative tissues from animals maintained on the scorbutic diet, both with or without supplementary ascorbic acid. The results are shown in table 1. The data given for guinea pigs and cotton rats are incomplete because of intercurrent deaths in the two groups of animals.

[2] Wesson.
[3] Hoffman-LaRoche.

It appears from this table that cotton rats, like albino rats, on an ascorbic acid-free diet from 17 to 51 days, were able to maintain normal vitamin C levels in their tissues, approximating those attained with the same diet supplemented by an additional intake of ascorbic acid. The slight drop observed in the adrenal of cotton rats fed the scorbutic diet is too small to be statistically significant. Moreover, none of the rats showed any symptoms of scurvy and all remained in good health throughout the entire course of the experiment. Data for only three guinea pigs receiving the scorbutic diet without supplementary ascorbic acid are available as the remainder of this group died of intercurrent infections. As might have been expected, these figures revealed a marked drop in the vitamin C levels of all tissues examined; this drop was more than compensated for by the supplementary intake of ascorbic acid in the protected parallel group.

SUMMARY AND CONCLUSIONS

The results reported in this paper show that the ascorbic acid content of the liver, spleen and brain of the cotton rat stands at about the same level as that of corresponding tissues of the albino rat. These concentrations are, of course, considerably above those of the guinea pig. However, the vitamin C level of the adrenal in cotton rats is markedly below that in albino rats and begins to approach the figures for guinea pigs. In spite of this relatively low adrenal storage, the cotton rat seems perfectly capable of maintaining, throughout its tissues, a normal threshold of vitamin C when deprived of ascorbic acid in its diet. Moreover, an additional intake of ascorbic acid is apparently not utilized since it fails to raise the vitamin C values beyond their existing limits for each organ. This would suggest that the cotton rat, like the albino rat, possesses some physiological mechanism for synthesizing vitamin C, in sharp contrast to the demonstrated inability of the guinea pig in this respect. The vitamin C metabolism of cotton rats may therefore be said to compare essentially with that of albino rats, save for the relatively

low adrenal storage of ascorbic acid, which resembles somewhat the characteristics of the guinea pig.

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A COMPARISON OF THE NUTRITIVE VALUES OF RAW, PASTEURIZED AND EVAPORATED MILKS FOR THE DOG [1]

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THREE TEXT FIGURES AND ONE PLATE (SIX FIGURES)

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The wide use of commercially processed milks in infant nutrition has raised several questions concerning the nutritive value of these milks in comparison with the original raw milk. It is still impossible to compare on a quantitative basis all the nutrients present in the various milks because assay procedures are not sufficiently perfected. Elvehjem, Hart, Jackson and Weckel ('34) compared the nutritive value of raw and pasteurized milks by following the growth of rats on these milks after mineralization with iron, copper, and manganese. When the experiments were conducted for only 6 weeks, greater differences were observed between winter and summer produced milks than between raw and pasteurized milks. Other animals, including swine (Kemmerer et al., '32) and dogs (Potter et al., '38), have been maintained for long periods of time on mineralized raw milks. Recently Evans and Phillips ('39) raised rats through five generations on milk supplemented with iron, copper, and manganese.

We felt that if differences did exist between various milks it might be possible to demonstrate such differences through

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reproduction experiments with dogs. The results reported in this paper must be considered preliminary in nature both because of the limited number of dogs used and because no attempt was made to control the source of the milk used for the processed products. Nevertheless the requirements of dogs are evidently sufficiently divergent from those of rats to demonstrate certain differences in the various milks.

EXPERIMENTAL

The original experiment was set up with one male and one female dog on each of the following milks: raw, obtained from several cows in the university herd maintained on a standard ration [2] throughout the experiment, pasteurized and evaporated (non-irradiated). The animals were fox terrier litter mates and were started on the milk diets when 9 weeks of age. They were kept in galvanized wire pens on a dry concrete floor covered with shavings. The daily mineral supplement per dog included 5 mg. of Fe as the pyrophosphate, 1 mg. Cu as the sulfate and 1 mg. Mn as the chloride and was added to a small amount of milk for the morning feeding to insure complete consumption. The evening feeding of milk was large enough to allow ad libitum consumption. Cod liver oil was added to the ration of all the dogs at the points designated on the growth curves.

The dogs were weighed weekly and the following blood determinations were made periodically: calcium, inorganic phosphorus, hemoglobin, cell volume, and cell count. No significant differences were observed between the groups. Data for calcium and phosphorus on the individual dogs have been published separately (Anderson and Elvehjem, '40). The young obtained from the original pairs of dogs were used as additional animals on the various milks.

Raw milk. The growth curves for the original pair of dogs on raw milk are given in figure 1. Grossly there has never

[2] The winter ration consisted of corn silage, timothy hay, and a grain mixture of corn and oats. For some of the cows part of the grain mixture was replaced by urea or linseed oil meal as a nitrogen source. Milk from these cows was consistently low in the grass juice factor.

been any indication that these dogs were suffering from a nutritional deficiency (figs. 1 and 2, pl. 1). However, the response in blood calcium and inorganic phosphorus when cod liver oil was administered (Anderson and Elvehjem, '40) indicates that there was a borderline deficiency of vitamin D. From point A in figure 1 the dogs were given 30 International units of vitamin D per day and at point B the level was increased to 125 I.U. per day.

At the point "D" dog 5 gave birth to five pups. Of these, two died within 18 hours, and another the following week. One of the pups was accidentally killed by the dam about the time of weaning. The remaining pup grew rather slowly in com-

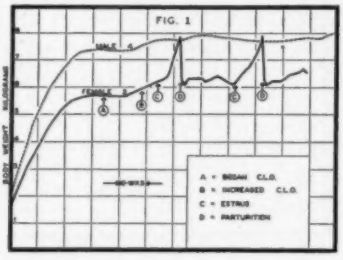

Fig. 1 Growth curves for dogs on raw milk, beginning the ninth week post partum. Dog 5 had litters of five pups each time.

parison to the original litter but otherwise showed no gross evidence of a nutritional deficiency. This pup was maintained on the raw milk ration with added cod liver oil and minerals for a year, and was then transferred to another experiment.

At the next point "D" dog 5 gave birth to a litter of five pups, one of which died at birth. The others, apparently vigorous pups, grew well showing no signs of a nutritional deficiency.

In a previous report (Anderson et al., '39) we described a deficiency in dogs on the evaporated milk ration which responded to vitamin E therapy. To ascertain whether or not the young dogs on the raw milk were deficient in this factor, two pups from this litter were given 5 mg. of dl-α-tocopherol-

acetate [3] per dog per week. The other two dogs were used as controls. The growth of the animals in the two groups was very similar and no dystrophy [4] was evident. Two of these dogs have been continued on the raw milk diet and have been bred in order to determine if the third generation can be reared successfully.

Evaporated milk. Figure 2 gives the data on the original pair of dogs maintained on the mineralized evaporated milk plus cod liver oil. The male (dog 2) showed no indication of nutritional deficiency (fig. 3, pl. 1) until the dog has been on experiment for 2 years. At this time (point C on curve) this

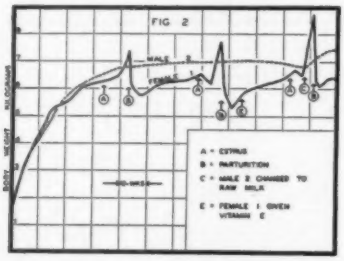

Fig. 2 Growth curves for dogs on evaporated milk, beginning the ninth week post partum. Dog 1 had litters of three, five and six pups respectively. Note the increase in body weight of dog 2 when changed to a diet of raw milk.

dog was transferred to raw milk and showed an immediate increase in body weight. Since this increased weight has been maintained for a period of 5 months and the level is significantly higher than the weight level he maintained on the evaporated milk, it would appear that the evaporated milk was not quite optimal for growth and maintenance of the male dog.

[3] We are indebted to Hoffmann La Roche, Inc., Nutley, New Jersey, for generous supplies of synthetic dl-α-tocopherol-acetate.

[4] The term muscle dystrophy is used in this paper to designate the general muscular weakness. Since we have treated most of the dogs showing any muscular weakness with vitamin E, we do not have sufficient material for extensive histological studies.

The female (dog 1) has had three litters of pups to date. The muscle dystrophy occurring in the pups of the first two litters has already been described (Anderson et al., '39). Two of the dogs in the second litter were protected from dystrophy by the administration of vitamin E (figs. 4 and 5, pl. 1). The condition of dog 1 during the second period of lactation was also described; and due to the severe skin lesions on the dam and the rapid loss of weight, the young were placed on mineralized evaporated milk on the eighteenth day of age. Definite muscle dystrophy did not develop in these dogs but the growth response obtained when dl-α-tocopherol was added indicated that vitamin E was still a limiting factor.

In order to determine if a completely normal litter could be produced by giving vitamin E during pregnancy, dog 1 was given 10 mg. of the tocopherol acetate per week beginning at point E and increased to 5 mg. per day during gestation. A very striking difference was observed between this gestation period and the former one with regard to the weight increase. In previous gestations the animal's weight had not increased in the last 5 days before parturition and in both cases she had lost weight during the last 2 days before parturition. In this gestation, when she received the added vitamin E, her weight continued to increase until the day of parturition. She also gained a kilogram more in weight than in the previous gestation yet gave birth to only one more pup.

The pups were somewhat larger than those of former litters yet all died within 3 days. Externally the pups showed a considerable number of lesions on the legs, joints, lower jaw and abdomen. The lesions varied in diameter from 1 to 8 mm. Some had formed scabs; others were apparently open lesions. From gross examination these lesions were quite similar to those which developed on the dam in the previous lactation period. Since the pups did not survive, it is impossible to determine if the added vitamin E would have given protection against the muscle dystrophy.

Autopsies on the pups showed considerable amounts of free blood in the abdominal cavity. Similarly hemorrhagic areas

were observed in the pericardium, lungs, and subcutaneous areas. In many cases these hemorrhages were of such severity that they gave a decided blue black color to the area as early as 24 hours before the death of the animal. Perhaps the most striking observation was the fact that the intracranial blood vessels were severely engorged. In three cases they had ruptured and severe hemorrhages had resulted.

Bacteriological examinations of the organs all showed negative results, and histological studies confirmed these observations since there was no apparent infection but rather a generalized congestion and extensive hemorrhage of the blood vessels.

Pasteurized milk. Because results indicated that the pasteurized milk had a nutritive value between that of the raw and the evaporated milks, photographs and growth curves will not be included in this report.

In the first litter of pups born to dog 3 on pasteurized milk there was no indication of a nutritional deficiency. In the second litter, however, of the three pups born, two died within a week. Both showed the hemorrhagic condition which has been described above for the pups on evaporated milk. It would appear then that evaporated and pasteurized milks are somewhat alike in this respect.

The only pup to survive in the second litter of dog 3 was nourished entirely by the dam up to the time of weaning. At this time the pup showed many of the early signs of muscle dystrophy. Most striking was the lack of muscle tonus in the feet. Between the femur and tibia the weakness was not so striking as that in the pups on the evaporated milk but the atony of the feet was the most severe case which we have thus far encountered.

When this pup was weaned and transferred to mineralized pasteurized milk the condition improved. Hence it would appear that the pasteurized milk contains enough vitamin E to prevent muscle dystrophy in the growing dog although not an adequate amount for pregnancy and lactation.

Food consumption. Figure 3 summarizes the results of the food consumption records for the dogs. No differences occurred between the groups on raw, pasteurized and evaporated milks which might be attributed to the nutritive value of the milk. A closer correlation seemed to exist between the amount of milk consumed and the relative activity of the animal. The least active dog required 17.7 cc. of milk per gram increase of body weight, while the most active dog required 21.2 cc. Average values for the group showed that for the first 12 weeks the dogs required approximately 20 cc. of milk per gram increase of body weight. This amounts to 2.5 gm. of milk solids per gram increase of body weight.

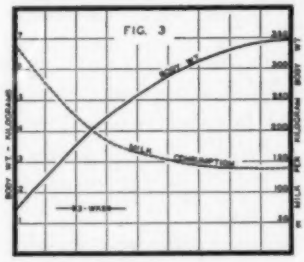

Fig. 3 Curves showing the amount of milk consumed per kilogram of body weight during the period of rapid growth.

DISCUSSION

It is apparent that whole raw milk supplemented with iron, copper, manganese, and cod liver oil will allow normal growth and reproduction in dogs. However, when pasteurized or evaporated milks are used there seems to be a limiting intake of at least two factors. One factor is related to the prevention of muscle dystrophy since all pups born to the female on evaporated milk showed definite dystrophy if they lived until 20 days of age. Since vitamin E produced beneficial effects when therapy was started early enough, the factor concerned must either be vitamin E or closely associated with it. The results indicate that pasteurized milk carries more of this factor than the evaporated milk.

It is interesting that the hemorrhagic condition did not develop in the pups until the production of the third litter in the case of the dog on evaporated milk and the second litter in the case of the animal receiving pasteurized milk. There is some possibility that the hemorrhagic condition may be related to vitamin K. The vitamin K content of the various milks is now being studied.

Herman ('36) has described a hemorrhagic condition in the kidneys, lungs, thymus, and heart of calves after 12 to 15 months on mineralized milk. Since he also used mineralized milk supplemented with cod liver oil, the same dietary factor may be involved. The dog may have a smaller requirement since no hemorrhagic condition was noted in the pups from the dog on raw milk.

All of the animals in our colony have had very excellent hair coats. Only under the strain of lactation with the dog on the evaporated milk and her dystrophic pup were there any exceptions to this condition. The excellent condition of the fur may be related to the high fat content of the milk when expressed on the dry basis.

It is impossible to apply the results obtained in this study directly in infant nutrition. Infants are rarely restricted entirely to milk diets and certainly the period of milk feeding is not comparable to the length of time that these experiments were conducted. However, the results do show that repeated pregnancy and lactation periods may bring out nutritional deficiencies which cannot be demonstrated by growth experiments alone. It should also be emphasized that the same type of pasteurized and evaporated milks were used throughout the experiment and that the results obtained with these milks may not be true for all pasteurized and evaporated milks.

SUMMARY

1. Whole raw milk supplemented with iron, copper, and manganese, and cod liver oil, allowed good growth and reproduction (two gestations) in fox terrier dogs. It was necessary to add cod liver oil in order to maintain a normal blood calcium and phosphorus level.

2. When evaporated milk was fed under similar conditions most of the young born showed either muscle dystrophy or a hemorrhagic condition of the pericardium, lungs, and brain. Vitamin E was effective in curing or preventing the muscle dystrophy but did not prevent the hemorrhagic condition.

3. The young born to the female on fortified pasteurized milk showed some signs of dystrophy and hemorrhage, but the symptoms were not as severe as those observed in the case of evaporated milk.

4. These deficiencies were only produced by repeated pregnancy and lactation since all the milks supported good growth of the males for almost 3 years and maintained the females in good condition except under the added strain of reproduction.

ACKNOWLEDGMENT

We are greatly indebted to Mr. J. J. Tamulis for valuable assistance in the care of the animals and to Mr. James H. Shaw for the histological studies.

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PLATE 1

EXPLANATION OF FIGURES

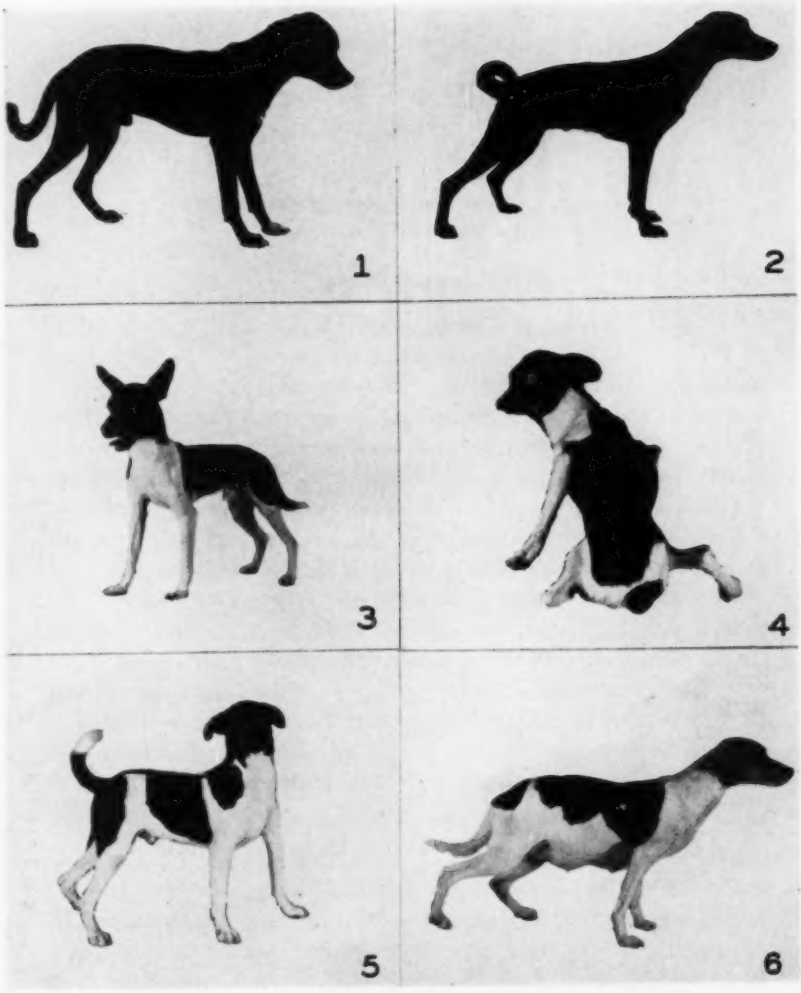
1 and 2 Dogs 4 and 5 raised on raw milk, normal after 2½ years.

3 Dog 2, raised on evaporated milk for 2½ years.

4 Dog from first litter produced by dog 1 (on evaporated milk). Pup was maintained on the fortified evaporated milk after weaning. Photograph taken when dog was 16 months old.

5 Dog from second litter produced by dog 1 (on evaporated milk). Pup was maintained on the fortified evaporated milk after weaning but 5 mg. of dl-α-tocopherol-acetate was added per week. Photograph taken when dog was 7 months old.

6 Dog 1 after the third gestation period in which she received 5 mg. vitamin E per day. Since the pups died, she was not subjected to the strain of lactation.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EFFECT OF CHOLINE AND OTHER SUPPLEMENTS ON PEROSIS

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TWO FIGURES

(Received for publication August 10, 1940)

Perosis, or "slipped tendon," has been extensively studied with chicks. The work, which includes many studies of the preventive effect of manganese, has been reviewed (Jukes, '37; Wilgus et al., '39). Recently it was observed (Jukes, '39 a) that turkeys were highly susceptible to perosis, and that additions of manganous sulfate to the experimental diet up to a level corresponding to 1440 parts of manganese per million of diet slightly accelerated the onset of this nutritional deformity. Injections of manganese were also ineffective. Later it was reported (Jukes, '40 a) that choline was effective in preventing perosis in turkeys.

Ringrose, Martin and Insko ('39) reported a lowering of the incidence of perosis in turkeys by the addition of manganese to an undescribed diet. Complete protection was not obtained.

EXPERIMENTAL

Turkeys were placed in electrically heated battery brooders at hatching and were fed the experimental diets immediately. From ten to fifteen birds were used in each group. Examination for perosis was made as previously described (Jukes, '40 a).

The appearance of affected turkeys corresponded to the symptoms of perosis in chicks as outlined by the studies of

Wilgus et al. ('37), Gallup and Norris ('38), and Caskey et al. ('39). Figure 1 illustrates the leg bones from perotic and non-perotic turkeys, and is strikingly similar to figure 1 of Caskey et al. ('39).

In a preliminary series of experiments, the diet formerly employed (Jukes, '39 a) was used. It consisted of the following in percentage composition: yellow cornmeal, 63.5; dried skim milk, 15; washed casein, 13; alfalfa meal, 1; $CaCO_3$, 4; KH_2PO_4, 2; NaCl, iodized, 0.5; and fish oil blend (3000-A,

a b c d e f

Femora Tibiae Metatarsi

Fig. 1 Leg bones (a, c, e) from a turkey receiving a supplement of a synthetic mixture containing choline compared with bones (b, d, f) from a turkey on basal diet 161. The former bird weighed 207 gm., and the latter, 205 gm. when they were killed at 4 weeks of age.

400-D), 1. The following five modifications of the diet were all found to be completely ineffective in preventing perosis: (1) Substitution of limestone and bonemeal for $CaCO_3$ and KH_2PO_4 respectively; (2) replacement of 25 parts of yellow cornmeal by wheat bran; (3) addition of 2 parts of wheat bran ash to 100 parts of diet; (4) addition of 0.5 part of mineral mixture (consisting of ZnO, 5; $Al_2(SO_4)_3$, 5; $CoCl_2$, $2H_2O$, 1; ferric citrate, 89) to 100 parts of diet; (5) omission of KH_2PO_4 and halving the $CaCO_3$ content.

In succeeding experiments the diet above was modified slightly to contain 65 parts of yellow cornmeal, 3 parts of $CaCO_3$, 1 part of KH_2PO_4 and 0.1 part of $MnSO_4$ (diet 161 (Jukes, '39 a)). The other ingredients were unchanged. In each series a "positive control group" was included on a normal diet, consisting of diet PD (Jukes and Sanford, '39), 99.5 parts, and fish oil blend (3000-A, 400-D), 0.5 part. Birds which died during the first 9 days were omitted from the calculations since perosis seldom developed before the ninth day.

In the first series some natural foods were tested with the results indicated in table 1.

TABLE 1

CHANGES IN DIET 161	PER CENT OF BIRDS SHOWING PEROSIS AT:				
	12 days	14 days	19 days	29 days	33 days
None	36	64	82	100	100
Ground wheat replacing yellow cornmeal	40	70	80	80	80
15 parts alfalfa meal replacing 15 parts yellow cornmeal	36	64	64	73	73
5 parts dried pork liver replacing 5 parts casein	36	45	45	36	27
Normal diet replacing diet 161	0	0	0	0	0

Wheat and alfalfa possibly exerted a slight anti-perotic effect. The effect of alfalfa was so low that vitamin K was excluded as an anti-perotic substance. In the case of liver, there was a tendency for some of the milder cases to retrogress after the third week.

Recently Hogan, Richardson and Patrick ('40) announced that liver contained a water-soluble organic factor which prevented perosis in chicks. This prompted an examination of the known B-vitamins. A mixture ("synthetic vitamin mixture") was made which included thiamin, 1.2 mg.; riboflavin, 1.8 mg.; nicotinic acid, 3 mg.; pyridoxine (vitamin B_6), 0.9 mg.; and pantothenic acid, 10 mg. To this mixture was added choline chloride, 0.15 gm., since this substance had been observed to stimulate growth slightly in chicks (Jukes, '40 b). The results are summarized in table 2.

The synthetic mixture completely protected against perosis. Sardine meal also appeared to supply a small amount of a protective factor which caused retrogression of mild cases after the third week, as was observed in the case of liver in the first series.

In the next experiment, thiamin, nicotinic acid, pantothenic acid and choline were tested separately. Only choline was effective. This experiment was previously reported (Jukes, '40 a). Some other supplements were tested in the same series with the results indicated in table 3.

TABLE 2

CHANGES IN DIET 161	PER CENT OF BIRDS SHOWING PEROSIS AT:				
	12 days	14 days	19 days	24 days	28 days
None	33	44	67	78	89
Synthetic vitamin mixture plus choline, as above, added to 100 gm. diet	0	0	0	0	0
17 parts sardine meal replacing casein and $CaCO_3$	20	60	70	30	10
Normal diet replacing diet 161	0	0	0	0	0

TABLE 3

SUPPLEMENT TO 100 GM. OF DIET 161	PER CENT OF BIRDS SHOWING PEROSIS AT:			
	12 days	14 days	19 days	28 days
None	71	71	85	85
Barley replacing cornmeal	12	12	12	38
2 gm. fish oil blend (3000-A, 400-D)	40	50	60	60
10 gm. gum arabic + 5 gm. gelatin	43	57	71	86
10 gm. dehydrated cane molasses	25	25	25	50
Normal diet replacing diet 161	0	0	0	0

At this time it was noted that there was no mortality during the first week in the case of a group of birds receiving supplementary thiamin. In succeeding experiments, diet 161 was modified by the addition of 50 μg. of thiamin to 100 gm. of diet.

Table 3 shows that barley supplied a small amount of the protective factor, but that additional vitamins A and D had no effect. A combination of gum arabic and gelatin, shown by Almquist, Mecchi, Stokstad and Manning ('40) to supply factors essential for the growth of chicks, did not prevent

perosis. Cane molasses was fed as a source of biotin. A sample of the molasses was kindly assayed by Dr. E. E. Snell and found to contain 2.6 μg. of biotin per gram. The preventive action of the molasses was very low.

In the next experiment a number of synthetic substances were tested, including betaine, which has been shown to have certain biological activities similar to those of choline (Best and Huntsman, '32; du Vigneaud et al., '39). Arseno-choline [1] was tested for the same reason (Welch, '36). The results are in table 4.

TABLE 4

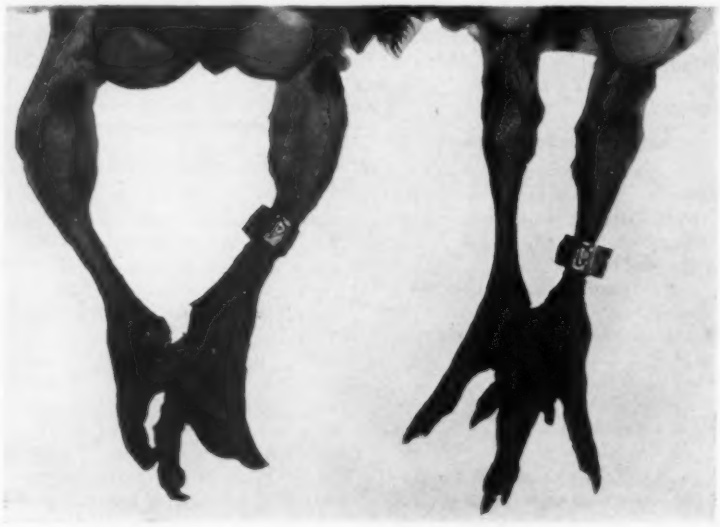
SUPPLEMENT TO 100 GM. OF DIET 161	PER CENT OF BIRDS SHOWING PEROSIS AT:				
	11 days	15 days	21 days	23 days	28 days
None	20	20	50	50	50
Synthetic vitamin mixture as above, without choline	25	42	58	50	50
0.2 gm. choline	0	0	0	0	8
Synthetic vitamin mixture with 0.2 gm. choline	0	0	0	0	0
0.2 gm. choline + 24 mg. pantothenic acid.	0	0	9	9	0
24 mg. pantothenic acid	50	40	60	60	60
0.9 mg. pyridoxine	18	55	64	64	82
0.2 gm. arsenocholine	0	9	9	9	9
0.5 gm. betaine	64	82	82	82	82
25 gm. rice bran replacing 25 gm. corn	11	11	22	22	11
25 gm. wheat bran replacing 25 gm. corn	25	33	58	50	50
25 gm. soybean meal replacing 12 gm. corn and 13 gm. casein	9	0	0	0	0
Normal diet replacing diet 161	0	0	0	0	0

The weights refer to choline and arseno-choline as the chlorides, and to betaine as the hydrochloride in all experiments in which these compounds were used. The results indicated that choline alone of the components of the synthetic mixture was able to prevent perosis. Arseno-choline was definitely effective, although one mild case of perosis appeared. Betaine was completely ineffective at the level fed. Of the natural foods, soybean meal was a relatively good source of the

[1] Kindly supplied by Dr. A. D. Welch.

preventive factor, rice bran was a poor source, and wheat bran was ineffective. Figure 2 illustrates a case of perosis from this series.

In the preceding experiments it was observed that choline as a sole supplement was effective in preventing the gross symptoms of perosis. However, when choline was fed in combination with the synthetic vitamin mixture, the leg bones



a b

Fig. 2 Perotic hock joints (a) of a turkey receiving betaine (see table 4) compared with normal hocks (b) of a turkey receiving choline as a supplement to diet 161. The birds were 21 days old when photographed, and weighed 107 and 147 gm. respectively.

were more normal in appearance than when choline was fed alone. This led to the adoption of a more "simplified" basal diet, containing yeast as a source of the vitamin B complex. It was found that choline prevented perosis and also promoted growth when fed as a supplement to this diet (Jukes, '40 a) which consisted of glucose,[2], 50 parts; washed casein, 30; dried

[2] "Cerelose."

yeast,[3] 10; salt mixture (Jukes, '39 b), 5; soybean oil, 4; and fish oil blend, 1 (diet 162). The diet contained approximately 166 parts of manganese per million parts of diet. The results shown in table 5 were obtained.

The results in table 5 indicate that 0.1% of choline was a sufficient supplement for growth but was not enough to prevent perosis completely. There was a tendency for some of the milder cases to retrogress in this group during the latter half of the experimental period. This recalls the preceding results with partially protective diets. The addition of 0.2% of

TABLE 5

Incidence of perosis and growth on a simplified diet

SUPPLEMENT TO 100 GM. OF DIET 162	PER CENT OF BIRDS SHOWING PEROSIS AT:					WEIGHT IN GRAMS AT:			
	10 days	12 days	13 days	15 days	28 days	7 days	15 days	19 days	28 days
None	27	36	55	73	82	62	90	110	155
0.1 gm. choline	8	8	33	33	17	62	120	158	211
0.2 gm. choline	0	0	0	0	0	62	110	137	209
0.1 gm. choline + 5 gm. gum arabic + 5 gm. gelatin	0	11	22	33	33	62	114	154	211
0.3 gm. betaine	25	62	75	88	100	61	84	106	139
20 gm. rice bran	50	70	80	70	80	68	109	143	189
Normal diet replacing diet 162	0	0	0	0	0	65	119	159	208

choline completely protected against perosis and produced bones of normal appearance. Betaine was ineffective and the symptoms in the birds receiving betaine were more acute than in the birds on the basal diet. Gum arabic and gelatin did not produce additional growth, thus suggesting a species difference between chicks (Almquist et al., '40) and turkeys. Twenty per cent of rice bran improved growth but was ineffective in preventing perosis. This is in disagreement with the result obtained with rice bran on diet 161. Perhaps this was due to diet 162 being more deficient than diet 161, and

[3] Anheuser-Busch, strain G.

hence the slight anti-perotic activity of rice bran was too weak to become apparent in the case of the more deficient diet.

In another experiment a group of nine turkeys were injected weekly with 0.1 gm. inositol, which was the level used by Wiese et al. ('38) in experiments with chicks, and reported by them to prevent perosis. However, perosis developed in the injected turkeys at approximately the same rate as in the case of birds on the basal diet.

AN EXPERIMENT WITH CHICKS

The preceding observations naturally raised the question of the possibility of an effect of choline on perosis in chicks. A somewhat different diet was devised to test this point.

TABLE 6

Effect of choline on growth and perosis in chicks

SUPPLEMENT TO 100 GM. OF BASAL DIET 109	PER CENT OF BIRDS SHOWING PEROSIS AT:				WEIGHT IN GRAMS AT:			
	12 days	17 days	25 days	32 days	8 days	17 days	25 days	32 days
None	33	33	50	75	55	78	116	145
0.1 gm. choline	0	0	0	9	54	95	141	175

Sodium alginate [4] and gelatin were added to supply the "rice factor" (Almquist et al., '40). Synthetic vitamins were used to replace part of the yeast. The diet had the following percentage composition (diet 109): glucose,[5] 53 gm.; washed casein, 25; gelatin, 5; sodium alginate,[4] 5; soybean oil, 5; dried yeast,[6] 5; salt mixture (Jukes, '39 b), 6; fish oil blend, 0.3; thiamin, 0.6 mg.; riboflavin, 0.8; nicotinic acid, 1.5; pyridoxine, 0.6; pantothenic acid, 3.0. Twelve Single Comb White Leghorn chicks were used in each group. The results are summarized in table 6, and indicate that choline is essential for the prevention of perosis in chicks.

[4] "Kelgin."
[5] See footnote 2.
[6] See footnote 3.

Growth was promoted by choline, but the stimulatory effect was less in the case of the chicks than was observed with turkeys (table 5).

DISCUSSION

Dietary choline has been demonstrated to have the following actions: lipotropic (Best et al., '32); protective against hemorrhagic degeneration of the kidney (Griffith and Wade, '39); and functioning in the conversion of homocystine to methionine (du Vigneaud et al., '39). In all three cases, betaine has an action similar to that of choline (Best and Huntsman, '32; Welch, '40; du Vigneaud et al., '39). This might indicate that choline is the precursor of betaine and that betaine, formed from choline in vivo, is actually responsible for the physiological activity of dietary choline. However, it has been suggested by Welch and Welch ('38) that betaine functions in this regard as a precursor of choline rather than the reverse being true. The complete lack of growth-promoting and anti-perotic activity of betaine indicates that the turkey is unable to convert betaine to choline to an appreciable extent. This further suggests that betaine is not formed from choline in vivo as a necessary step for the physiological action of choline, and that in cases where dietary betaine is active, the activity is due to the ability of the experimental animals (rats) to convert betaine to choline. Evidently turkeys do not possess this ability to an effective degree.

No biochemical explanation of perosis has yet been advanced. Perosis in chicks has been found to be associated with a slight lowering of the ash content of the bones (Caskey et al., '39). It has also been stated (Wiese et al., '38) that the phosphatase content of the blood and bones of perotic chicks is lower than normal. The present investigation shows that choline is necessary for normal bone formation in chicks and turkeys. Choline is known to accelerate the formation of phospholipids in the liver, following which the phospholipids are rapidly removed from this organ (Perlman and Chaikoff, '39). This observation links choline with phosphorus metabolism. The formation of

phospholipids would presumably be accompanied by increased esterification of phosphoric acid. This might lead to a drain upon the supply of inorganic phosphate. It is interesting to note that Nielsen and Madsen ('40) reported that the serum inorganic phosphorus of perotic turkeys was higher than normal.

It is possible to speculate upon the question of the identity of choline with the anti-perotic factor in feeding stuffs. Choline is present in lecithin and other phospholipids, and possibly also may be present in other forms (Thannhauser et al., '39). Some data are available on the lecithin content of some natural materials. According to data cited by Horvath ('36), soybeans contain from 1.6 to 3.0% of phosphatides, and 38% of the phosphatide content is lecithin. A relatively small proportion of the phosphatides is removed by extraction of the oil by the usual methods (Horvath, '37). Soybean meal may hence be expected to contain about 0.6 to 1.1% of lecithin, which represents a content of about 0.08 to 0.15% of choline in soybean meal. The observed anti-perotic activity of soybean meal, noted also by Funk and Kempster ('40), may therefore be at least partially attributable to its choline content. Sardine meal, which had some preventive effect, would presumably furnish appreciable amounts of lecithin. Bähr and Wille ('31) reported that fish meal, prepared from whole fish, contained from 1.0 to 2.5% of "lecithin." Dried pork liver (table 1) was also observed to have some anti-perotic activity. Bloor ('28) reported the average lecithin content of fresh beef liver to be 1.6%. This would correspond to about 0.6% of choline combined as lecithin in dried beef liver.[7] Dried skim milk, used in basal diet 161, obviously had little or no anti-perotic activity. It is also low in lecithin. Heinemann ('39) reported that dried skim milk contains about 0.16% of lecithin.

Fresh egg yolk contains about 10% of phospholipids, and a sample of phospholipids prepared from egg yolk was found to consist of about 80% of lecithin (Jukes, '34). A consider-

[7] Fletcher et al. ('35) reported 0.27% of choline in beef liver, and 0.09% in dried skim milk.

able portion of the yolk is retained in the yolk sac within the body of the newly-hatched bird. The results in table 5 show that the birds on the basal diet grew at the same rate as the birds on the diets supplemented with choline in the first week, after which a difference appeared in the rate of growth. This would indicate that the turkeys were utilizing a reserve supply of choline during the first week. In this connection it may be noted that the yolk sac of the newly hatched chick contains 1.6% of phospholipid, nearly all of which is absorbed by the chick during the first week (Entenman et al., '40).

SUMMARY

1. The addition of choline to a deficient basal diet, containing added manganese, prevented perosis in turkeys. Arsenocholine was also anti-perotic, but betaine was completely ineffective.

2. A number of vitamins and minerals were tested and all were found ineffective. However, an adequate supply of the vitamin B complex in the diet was found to be necessary for the full anti-perotic effect of choline to be exerted.

3. The anti-perotic effect of various feeding stuffs was studied. Soybean meal was fully effective at a level of 25%. Partial protection was obtained with either 17% of sardine meal, 5% of dried pork liver, or with 65% of barley. Some other feeds had a slight potency. The relation of the lecithin content to the anti-perotic activity of certain feeds is discussed.

4. Experiments with a simplified diet indicated that choline is essential for growth as well as for the prevention of perosis. An addition of 0.1% of choline was sufficient for growth, but insufficient for the prevention of perosis. An addition of 0.2% of choline protected against perosis. Betaine was neither growth-promoting nor anti-perotic. Inositol was ineffective.

5. The addition of a supplement to supply the "rice factor" did not promote growth in turkeys, which may indicate a difference between this species and the chick.

6. An experiment with chicks indicated that choline is essential for the prevention of perosis in this species also.

7. Biochemical possibilities arising from these observations are discussed briefly.

The assistance of Robert E. Ranney and Phillip Levy is gratefully acknowledged. Thiamin, riboflavin, nicotinic acid and pyridoxine were kindly supplied by Merck and Company. Dried yeast was generously furnished by Anheuser-Busch, Inc., and washed casein by the Golden State Company.

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THE DISTRIBUTION OF THE "GRASS JUICE FACTOR" IN PLANT AND ANIMAL MATERIALS [1]

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ONE FIGURE

Previous publications from this laboratory have shown that young grass contains a water-soluble growth-promoting substance (or substances) for the rat (Kohler et al., '36, '37) and the guinea pig (Kohler et al., '38). It has also been shown that this factor is distinct from all other known nutritional essentials (Kohler et al., '37, '38). Cows grazing on good pastures or fed good quality roughage can transmit this factor to their milk (Johnson et al., '39). Johnson et al. have also shown that the grass juice factor can be preserved through ensiling processes. The guinea pig has proved to be a more sensitive test animal than the rat in assaying for this factor (Kohler et al., '38). It was found that guinea pigs fed mineralized winter milk alone lost weight and died within 3 to 10 weeks, whereas those animals receiving the same milk supplemented with small amounts of grass or grass juice grew at a normal rate.

Kohler, Randle and Wagner ('39 a) showed that the "grass juice factor" was precipitated from grass juice by the addition of acetone and could be removed from this precipitate by wash-

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from the American Butter Company, Abbott Laboratories, and the Works Progress Administration.

[2] Now with Cerophyl Laboratories, Kansas City, Missouri.

ing with acid acetone. A chloroform-amyl alcohol mixture removed the proteins from the grass juice and left the factor in the aqueous layer. When this deproteinated juice was treated with norite, the filtrate retained practically all of the original activity. The best concentrates were active when fed at a level of 40 mg. of solids per day. This represented a fiftyfold concentration.

Cannon and Emerson ('39) have reported on the dietary requirements of guinea pigs on purified diets. These diets, although supplemented with the other known vitamins, failed to support growth. They reported that supplements of lettuce or grass to these diets would give normal growth for considerable periods and believed their factor to be identical with the "grass juice factor." However, the growth they obtained was markedly inferior to that obtained in this laboratory when the winter milk diet or purified diet (Kohler et al., '39 b) was supplemented with grass.

This paper presents a study of the distribution of the "grass juice factor" in various plant and animal tissues.

EXPERIMENTAL

The feeding technique described by Kohler, Elvehjem and Hart ('38) was used. Fresh unpasteurized winter milk, obtained from cows maintained throughout the year on a ration of grain, corn silage and dry hay, was supplemented with a mineral mixture supplying 1 mg. of iron as ferric pyrophosphate, 0.1 mg. of copper as copper sulfate and 0.1 mg. of manganese as manganese sulfate per day. Unless otherwise stated, all the food materials used in these assays were obtained fresh, chopped in a food chopper, dried in a drying room (35 to 40°C.), ground, and stored in a refrigerator (5 to 10°C.) until fed. The food materials were fed to guinea pigs (275 to 325 gm.) as supplements to a small amount of milk in the morning. Later in the day, after the supplement was consumed, a larger dish of milk was placed in the cage to insure ad libitum feeding. The animals were kept in individual wire cages with raised screen floors.

In the earlier studies on the "grass juice factor" in this laboratory, orange juice was fed as a source of extra ascorbic acid. Later experiments (Kohler et al., '38) showed that guinea pigs consuming from 80 to 200 cc. of milk showed no improvement with the addition of orange juice. This is consistent with the finding that milk contains 2 to 2.5 mg. of vitamin C per 100 cc. and that 0.3 mg. of pure ascorbic acid prevents scurvy and supports good growth in the guinea pig. Animals live from 3 to 10 weeks on the basal milk diet, and have never shown any signs of scurvy upon autopsy.

DISCUSSION

Typical growth curves for animals receiving mineralized winter milk alone and the basal diet plus certain supplements are shown in figure 1. Curve 629 is the usual type for animals

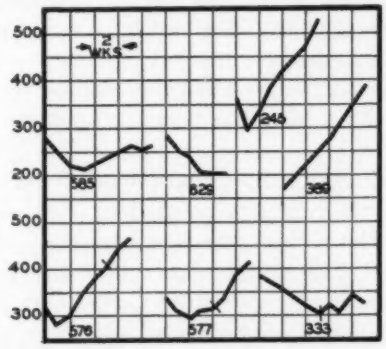

Fig. 1 Growth curves of guinea pigs fed mineralized winter milk plus 2 gm. of the supplement as follows:

585♂—Control
629♂—Control
245♀—Rye grass
389♀—Cerophyl no. 71921
576♂—Peas
577♂—Canned peas
333♂—Carrots
\ = supplement raised to 3 gm. per day.

receiving the basal ration; however, occasionally an animal is able to maintain itself for several weeks (curve 585). Under these experimental conditions, 2 gm. per day of a supplement rich in the grass juice factor promotes excellent growth in

guinea pigs (curves 245 and 389). Three grams per day does not increase the growth rate significantly over that of the 2 gm. level (curve 576). An increase in the amount fed per day may stimulate additional growth if the supplement is only a fair source of this factor (curve 577). Higher levels of a supplement that is poor in the "grass juice factor" do not increase the daily growth rate significantly (curve 333). It should be pointed out that there is usually a temporary loss in weight when the animals are shifted from the stock ration to the supplemented winter milk diet (fig. 1). Therefore, the average daily gain for the animals has been calculated on a 6 weeks' basis beginning with the second week.

A considerable number of plant tissues have been assayed for the "grass juice factor." The following have been found to be the best sources: dehydrated cereal grass,[3] rye grass, young white clover, peas, pea shells, cabbage, turnip tops, spinach, etc. The materials poorest in this factor were apples, celery, molasses, peanuts, turnips, lettuce, oats, etc. Such tissues as young berries, cauliflower, canned peas, beans, etc., were found to be intermediate sources of the "grass juice factor."

The data of this paper and other papers from this laboratory indicate that cereal grasses are excellent sources of the "grass juice factor." The results reported here support the observation made by Kohler et al. ('38) that the potency of the grasses as a source of this factor varies with the age of the plant, i.e., the small rapidly metabolizing plant is much richer than the older and more mature plant. The "grass juice factor" activity varies considerably from one plant to another (table 1). In addition, the amount of this factor in any one given plant material may also show a great variation.

Some of the crystalline vitamins and highly purified vitamin concentrates have been tested to determine whether they show a supplementary growth effect above that of the "grass juice factor." The following crystalline vitamins did not further stimulate growth in guinea pigs on a milk diet: ascorbic acid

[3] Cerophyl, supplied by the Cerophyl Laboratories, Kansas City, Missouri.

TABLE 1

Distribution of the grass juice factor in plant and animal tissues [1]

NO.	ANIMAL NO.	FOODSTUFF	LEVEL FED	AVERAGE WEIGHT GAIN	REMARKS
			gm./day	*gm./day*	
1	629	Control [2]	0	—1.3	
2	585	Control [2]	0	0.2	
3	245	Rye grass [2]	2	5.3	
4	389	Cerophyl [2]	2	4.9	
5	285	Clover, white	2	4.0	Young, 6–8 inches tall
6	286	Clover, white	2	2.0	Older, 8–10 inches tall
7	576	Peas	2–3 [3]	3.9	
8	601	Peas, frozen	2	4.0	Commercial
9	577	Peas, canned [2]	2–3 [3]	1.8	
10	575	Peas, pods	2–3 [3]	3.0	
11	605	Peas, juice	20 cc.	1.9	
12	604	Peas, shells	3	4.0	
13	607	Peas, pulp	2	1.1	Residue after pressing juice from peas
14	570	Bean, Ky. Wonder	2–3 [3]	3.0	
15	572	Beans, soy	2–3 [3]	3.0	Whole green beans
16	236	Carrots	2	3.0	
17	333	Carrots [2]	2	—0.8	
18	455	Young berries	2	1.3	
19	456	Young berries	13.3	1.4	Fed in fresh state
20	600	Lettuce	2	1.5	
21	599	Cabbage	2–3	4.0	
22	490	Turnips	2	0.8	
23	438	Turnip tops (greens)	2–3	3.9	
24	582	Spinach	2–3	3.6	
25	583	Cauliflower	2–3	3.3	Heart
26	584	Cauliflower	2–3	1.6	Outer leaves
27	400	Peanuts	2–5	1.1	
28	493	Apples	2	1.0	
29	478	Molasses (blackstrap)	4 cc.	0.6	
30	581	Celery	2–3	0.8	
31	621	Brewers' yeast	4	2.0	
32	409	Hulled oats	4	2.1	
33	552	Rolled oats	4	—2.0	
34	215	Sprouted oats	3	3.7	
35	263	Rice bran	2	2.3	
36	367	Wheat germ	2–4	1.4	
37	218	Brain	1	1.3	Dried
38	182	Egg white	2–5	0	
39	217	Egg yolk	2	—0.7	
40	535	Beef heart	2	—0.5	
41	620	Liver extract powder	2	0	Wilson's 1–20 powder [4]
42	617	Whole milk powder	2	—0.8	Klim
43	611	Skim milk powder	2.5	—8	
44	616	Whey powder	2	1.2	
45	207	Cerophyl no. 825—3 + 0.001 M KCN	2	4.9	
46	204	Cerophyl no. 825—3 + 1% H_2O_2	2	1.0	
47	208	Cerophyl no. 825—3 + NaOH	2	2.9	
48	206	Cerophyl no. 825—3	2	3.3	
49	205	Cerophyl no. 825—3 + H_2O	2	4.8	
50	209	Cerophyl no. 825—3 (ashed)	≎2	—1.6	

[1] The data given in this table represent the results of a typical response of one animal on each level of sample assayed. The actual number of animals used in each assay varied from one to six.

[2] Growth curves for animals receiving these supplements are included in figure 1.

[3] This indicates that the level of the supplement was raised during the course of the experiment.

[4] From the Wilson Laboratories, Chicago.

10 mg. per day, riboflavin 50 and 100 γ per day, vitamin B_6 50 γ per day, vitamin E 1 mg. per week, thiamin 500 γ per day and nicotinic acid 12.5 mg. per day. Highly purified concentrates of pantothenic acid (150 γ per day) and vitamin P preparations (citrin, hesperidin, and calcium eriodictate)[4] 1 mg. per day similar to those of Rusznyak and Szent-Gyorgyi ('36) failed to promote growth in the guinea pig on the basal ration.

Euler and Malmbery ('34) has reported the presence of an anti-pneumonia factor for guinea pigs in berries such as black currants, mountain ash berries, etc. Fresh frozen young berries[5] (both fresh and dry) were therefore investigated as a source of the "grass juice factor." They produced some growth (table 1).

Rygh and associates ('32) believed that in addition to ascorbic acid, narcotine or nor-narcotine was essential for the prevention of scurvy in the guinea pig. Since these alkaloids had been isolated from scorbutic rations, fruits and plant materials, narcotine hydrochloride[6] was fed to several animals at 30, 60 and 120 μg. per day without stimulation of growth. In one case, however, the animal on being raised to the 60 μg. level grew 3 gm. per day for 6 weeks, then exhibited a decline and died 5 weeks later. The growth in this case may have been due to a temporary stimulating effect of the alkaloid. Later ('38–'39) Rygh reported that glucuronic acid and narcotine prevented scurvy in guinea pigs. Ten milligrams of glucuronic acid[7] per day had no beneficial effects on guinea pigs deficient in the "grass juice factor"; a combination of glucuronic acid and narcotine was also ineffective.

Kohler, Elvehjem and Hart ('38) reported that the "grass juice factor" was labile to autoclaving or storage at room

[4] The citrin, hesperidin and calcium eriodictate were kindly supplied by Dr. C. Nielsen, Abbott Laboratories, North Chicago, Illinois.

[5] Birdseye brand.

[6] The narcotine hydrochloride was kindly supplied by Dr. M. H. Seevers, Department of Pharmacology, University of Wisconsin.

[7] The glucuronic acid was kindly supplied by Dr. K. P. Link, Department of Biochemistry, University of Wisconsin.

temperature. Further studies were made on the stability of this factor in grass. Three 120-gm. samples of dried grass (cerophyl no. 825-3) were weighed into beakers. One sample was treated with 300 cc. of 0.001 M potassium cyanide, another with 300 cc. of 1% hydrogen peroxide, and the third with cold sodium hydroxide solution, pH 9. The samples were allowed to stand at room temperature for 30 minutes. At this time the sodium hydroxide treated sample was neutralized and all three samples were dried at 35 to 40°C. for 24 hours. An untreated sample and a water-treated sample were fed at the same time as positive controls. Each sample was fed at a 2-gm. level. Still another portion of the grass was ashed, the ash suspended in water, and fed at the equivalent of the 2-gm. level. The water, potassium cyanide, and the sodium hydroxide treated samples were as effective in promoting growth as the untreated sample of grass. Hydrogen peroxide treatment destroyed practically all of the activity and the ashed sample retained none of the original activity. The destruction of the "grass juice factor" appears to be an oxidative process.

SUMMARY

The relative distribution of the "grass juice factor" in plant and animal tissues is reported. Animal tissues appear to be a poor source of the factor. The amount present in plant materials varies considerably. The young rapidly metabolizing plant tissues are much richer in this growth promoting substance than the older and more mature plant material. Much of the original activity can be retained by careful drying and storage at low temperatures. The factor is labile to oxidation. Glucuronic acid, narcotine, citrin and specific fractions of citrin, i.e., hesperidin, and calcium eriodictate, do not have the activity of the "grass juice factor."

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THE INFLUENCE OF LACTOSE ON CALCIUM RETENTION IN CHILDREN [1]

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The ability of lactose, the sugar of milk, to influence calcium metabolism or the ash content of body tissue has been demonstrated in several species—the dog (Dragstedt and Peacock, '23; Inouye, '24; French and Cowgill, '37), the rat (Bergeim, '26; Mitchell, Hamilton and Beadles, '37; Outhouse, Smith, Merritt and White, '37; Outhouse, Smith and Twomey, '38), the calf (Robinson, Huffman and Mason, '29), the chick (Kline, Keenan, Elvehjem and Hart, '32) and the pig (Whittier, Cary and Ellis, '35). In this laboratory, the increase observed in the total ash content of bones and in the calcium and phosphorus storage as a result of lactose-feeding was not as great as that obtained when vitamin D was administered in the form of cod liver oil. Kline, Keenan, Elvehjem and Hart ('32), who added lactose to the diet of irradiated chicks, suggested that a combination of this nutrient and vitamin D was more efficacious than either one administered alone; they believed that lactose might act as a supplement to vitamin D in promoting calcium assimilation. Such findings carry interesting implications as to the possibility of increasing calcium utilization in man—particularly since it has been shown that man is not economical in his use of dietary calcium (Steggerda and Mitchell, '39; Kinsman, Sheldon, Jensen, Bernds, Outhouse and Mitchell, '39; Kempster, Breiter, Mills, McKey, Bernds

[1] Aided by a grant from the American Dry Milk Institute, Inc., Chicago, Illinois.

and Outhouse, '40). To test this possibility, an experiment was devised which would show the effect, if any, of lactose on calcium metabolism in children; this paper is a report of that investigation.

EXPERIMENTAL

The subjects of this experiment were five little boys, four of whom had participated in the previously reported work on the utilization of the calcium of milk (Kinsman, Sheldon, Jensen, Bernds, Outhouse and Mitchell, '39) and of $CaHPO_4$ (Kempster, Breiter, Mills, McKey, Bernds and Outhouse, '40). Their ages, heights and weights are given in table 1. The basal dietary was the same as that of the previous studies; although it provided generous quantities of fruits, meat and eggs, it contained milk solids equivalent to only 200 cc. of fluid milk and, hence, was poor in calcium. Approximately 1000 I.U. of vitamin D in the form of cod liver oil were fed daily—and had been fed daily for many weeks prior to the beginning of the experiment—in order to provide optimal conditions for the utilization of calcium.

The source and quantity of calcium received by the five subjects during this experiment were essentially the same as those of period VI in the study on the utilization of the calcium of $CaHPO_4$. In fact, period VI, in which $CaHPO_4$ was given as a supplement to the basal dietary, constitutes the control period of the present study. (Inasmuch as subject D stored such small amounts of calcium during this period, a second control period, period IX, had to be run on this child.) During a subsequent period (period VIII) in which lactose was fed, this phosphate was administered but in a greater quantity; the increase was necessary in order to equalize the total intake of calcium for the two periods since the children had shown a tendency, during period VII,[2] toward a decrease in the consumption of ad libitum foods. The $CaHPO_4$ contributed 126 mg. of calcium during the 7 weeks of period VI

[2] Period VII was the basal period for the previously reported study on the utilization of diphosphate calcium and is not essential to the present report.

TABLE 1

Calcium metabolism data (ave. daily) during lactose and non-lactose feeding

PERIOD AND WEEK		INTAKE	OUTPUT		RETENTION
			Urinary	Fecal	
Subject W Age in yrs., 4.8				Weight in kg., 20.3 Height in cm., 108.6	
		mg.	mg.	mg.	mg.
VI	2[1]	479	37	338	104
	3	483	46	324	113
	4	509	58	323	128
	5	501	56	280	165
	6	497	60	296	142
	7	488	64	292	131
	Ave.	493	54	309	131
VIII	2	520	46	335	139
	3	511	58	280	173
	4	515	50	312	154
	5	514	55	317	142
	6	516	58	309	149
	Ave.	515	53	311	151
% retention: period VI, 26.6; period VIII, 29.3. Increase in % retention, 10.2%.					
Subject Jw Age in yrs., 7.0				Weight in kg., 20.7 Height in cm., 124.1	
VI	2	483	94	313	76
	3	489	123	303	63
	4	513	167	269	77
	5	493	139	308	47
	6	490	126	294	70
	7	484	151	265	79
	Ave.	492	133	292	69
VIII	2	518	110	271	137
	3	512	96	273	143
	4[2]	482	138	268	76
	5	520	95	320	106
	6	520	125	344	51
	7	514	108	286	120
	8	515	105	321	89
	9	518	85	314	119
	Ave.	517	103	304	109
% retention: period VI, 14.0; period VIII, 21.1. Increase in % retention, 50.7%.					
Subject W					
VII	2	346	65	171	110
	3	347	46	168	133
	4	357	81	216	60
	5	364	68	200	106
	6	369	79	167	123
	7	377	68	198	111
	8	355	50	195	109
	Ave.	359	65	188	107
X	2	341	50	191	100
	3	356	41	217	99
	4	349	34	235	80
	5	345	48	194	102
	Ave.	348	43	209	95
% retention: period VII, 29.8; period X, 27.3. Decrease in % retention, 8.4%.					

PERIOD AND WEEK		INTAKE	OUTPUT		RETENTION
			Urinary	Fecal	
Subject C Age in yrs., 5.9				Weight in kg., 22.6 Height in cm., 117.3	
		mg.	mg.	mg.	mg.
VI	2[1]	495	52	337	106
	3	504	57	356	90
	4	543	66	340	137
	5	523	75	344	104
	6	508	64	355	90
	7	496	72	334	91
	Ave.	512	64	344	103
VIII	2	522	60	351	112
	3	515	61	314	140
	4	516	57	411	48
	5	517	46	347	123
	6	517	35	351	131
	Ave.	517	52	355	111
% retention: period VI, 20.1; period VIII, 21.5. Increase in % retention, 7.0%.					
Subject Br Age in yrs., 6.8				Weight in kg., 26.4 Height in cm., 121.5	
VI	2	496	83	332	81
	3	511	118	297	97
	4	544	140	339	64
	5	523	109	320	95
	6	507	103	314	89
	7	488	101	319	69
	Ave.	512	109	320	83
VIII	2	520	90	293	136
	3	515	67	300	148
	4[2]	520	64	303	153
	5	533	66	283	185
	6	526	42	305	179
	Ave.	523	66	297	160
% retention: period VI, 16.2; period VIII, 30.6. Increase in % retention, 88.9%.					
Subject C					
VII	2	356	70	249	37
	3	364	77	225	62
	4	362	71	229	62
	5	371	63	256	52
	6	366	62	216	88
	7	376	69	218	89
	8	351	52	204	96
	Ave.	364	66	228	69
X	2	360	34	219	107
	3	375	29	199	147
	4	354	37	239	78
	5				
	Ave.	363	33	219	111
% retention: period VII, 19.0; period X, 30.6. Increase in % retention, 61.0%.					

Subject D.		Age in yrs., 6.1.		Weight in kg., 19.4.	Height in cm., 111.2.				
		Control period IX				Lactose period VIII			
	2[1]	517	56	312	149	484	55	311	117
	3	519	53	338	128	500	41	281	179
	4	515	59	299	156	505	46	305	154
	5	514	63	332	119	492	48	307	138
	6	513	54	292	167	500	47	312	141
	7	529	41	370	118				
	8[2]	487	47	376	63				
	9	511	53	337	121				
	Ave.	517	54	326	137	496	47	303	146
% retention: period IX, 26.5; period VIII, 29.4. Increase in % retention, 10.9%.									

[1] Data omitted for the first week following a change in the level of calcium intake.
[2] Subject ill; data not included in average.

and 176 mg. during the 6 to 9 weeks of period VIII. The total daily calcium intake for both periods approximated 500 mg.; this level was selected because, in previous studies, it had induced, in these same children, less than maximal retention of calcium. In studies on calcium utilization, the intake level is important inasmuch as the full effect of any substance could not be discerned if the subjects were already storing maximal quantities of calcium. As a matter of fact, as the experiment progressed it was suspected that subjects W and C were receiving too much calcium; therefore, during a subsequent short period (period X) they were given lactose in conjunction with the basal dietary. The retentions observed during this period were compared with those of period VII during which the basal dietary alone was fed.

Thirty-six grams of lactose supplemented the dietary during period VIII. This amount of the carbohydrate plus that of the milk solids of the basal dietary approximated the quantity of lactose contained in 1 quart of milk. The milk sugar was obtained from the same source as was that used in an earlier study of the influence of lactose on bone calcification in the rat (Outhouse, Smith, Merritt and White, '37); it was ash-free and contained no sterols. During this period, the quantity of sucrose which the children had been allowed in their basal diet was decreased by an amount equivalent to the added lactose so that the intake of di-saccharides might remain constant.

The procedures used in the collection of the metabolic materials and in the analysis for calcium corresponded exactly with those of the earlier experiments and need not be discussed here.

RESULTS

The children remained healthy, active and cooperative throughout the experiment and made satisfactory gains in weight. Their appetites were good, and ennui was never noted. During the period in which lactose was fed, no effect—adverse or otherwise—on intestinal motility was discerned.

The metabolic data are given in table 1. During the period in which $CaHPO_4$ was the only supplement to the basal dietary

(i.e., period VI for four subjects and period IX for subject D), the daily calcium intakes for subjects W, D, C, Br and Jw were 493, 517, 512, 512 and 492 mg., respectively; these values averaged 505 mg. At these levels of intake, the average quantity of calcium stored each day by the individual children, in the order listed above, was 131, 137, 103, 83 and 69 mg., respectively. These retentions amount to 26.6, 26.5, 20.1, 16.2 and 14.0% of the intake; the average for the group is 20.7%.

During period VIII when lactose was fed, the average daily intakes of calcium were 515, 496, 517, 523 and 517 mg., and the corresponding retentions were 151, 146, 111, 160 and 109 mg. for subjects W, D, C, Br and Jw. Expressed in terms of the per cent of the intake retained, these values become 29.3, 29.4, 21.5, 30.6 and 21.1 and average 26.4.

While subjects W and C were on the basal dietary (i.e., during period VII), they consumed, respectively, 359 and 364 mg. calcium daily and stored 107 and 69 mg. or 29.8 and 19.0% of the intake. During period X when 36 gm. of lactose supplemented the basal dietary, the children ingested 348 and 363 mg. of calcium and showed corresponding retentions of 95 and 111 mg. which represented 27.3 and 30.6%, respectively, of the intakes.

In this particular experiment, the criterion used for determining the effect of lactose on calcium metabolism involved a comparison of percentage retentions of calcium during the lactose period and the non-lactose period. A more desirable criterion would have been a comparison of percentage utilization values for these two periods, but the formula used in previous studies (Kinsman, Sheldon, Jensen, Bernds, Outhouse and Mitchell, '39; Kempster, Breiter, Mills, McKey, Bernds and Outhouse, '40) could not be applied since the lactose may have affected not only the di-phosphate calcium but that of the basal ration also. Inasmuch as each child did not consume exactly the same amount of calcium during both periods, the data had to be expressed in terms of the percentage increase in the percentage of calcium retained. On such a basis, the lactose fed during period VIII is shown to have

induced better calcium storage in all of these children; the retention percentages for the non-lactose and lactose periods, respectively, were 26.6 and 29.3 for W, 26.5 and 29.4 for D, 20.1 and 21.5 for C, 16.2 and 30.6 for Br and 14.0 and 21.1 for Jw. Though some of these differences may seem small, they constitute increases over the lower values of 10.2, 10.9, 7.0, 88.9 and 50.7% for the subjects in the same order as listed above. This would mean that there was an average increase in the amount of calcium retained during the lactose period of 33.5%. During period X when lactose was fed in conjunction with the basal dietary, subject C retained 61.0% more calcium than he did previously on the same low calcium intake when lactose was not fed whereas subject W responded conversely —retaining slightly less calcium.

In table 1 the data on pathways of excretion of calcium reveal a decrease in urinary values for all children during the time of lactose ingestion. The average losses in urinary calcium amounted to 16.9 and 12.0% of the intake during the control and lactose periods, respectively—a decrease of 29% during the latter period. (Statistical treatment of these data give high enough odds—i.e., 143 to 1—to enable one to conclude that the difference between the values was due to the ingestion of lactose and not to chance.)

DISCUSSION

The response of the subjects to the supplement of a moderate quantity of lactose during period VIII was unanimous; greater retentions of calcium were obtained in all cases. The mean increment in percentage retention for the group was approximately one-third of the mean percentage retention for period VI. Other evidence, which is fully as convincing of the stimulating effect of lactose on calcium utilization, can be secured for four of the subjects (subject D joined the group later in the experiment), by comparing data of periods II, III and IV during which the sources or levels of intake of calcium differed from those of periods VI and VIII. In every one of these periods, all subjects but one (Jw retained a higher percentage of calcium during one of these non-lactose periods)

retained an appreciably smaller percentage of calcium than they did later when they received lactose. If all the data for the three periods are averaged for each child or if the data for the four children are averaged for each period and then compared with the percentage retentions of the lactose period, the average increases in calcium retention during the lactose period are 35 and 42%, respectively, according to the two methods. These values check extremely well with the 39% increase over period VI observed for these four children and afford additional proof that lactose provided for a more efficient utilization of calcium by these children.

Although each child retained a greater quantity of calcium while ingesting lactose during period VIII, the response was far from uniform; the values ranged from 7 to 89%. The low values for subjects C and D (i.e., 7 and 10.2%, respectively) possibly are not truly representative of the influence which lactose can exert on the calcium metabolism of these two children. Subject C, at least, exhibited a 61% increase in per cent retention when he was given lactose in conjunction with a lower level of calcium intake, and subject D actually retained as much calcium during period VIII as he did during an earlier period when he received 1600 mg. calcium daily. The low value computed for the latter child is undoubtedly due to the fact that his control period followed the lactose period, and a portion of the large quantity of calcium which he stored then may have resulted, in part, from a persistence of the effect of lactose—which phenomenon occurred in all subjects but W—long after this sugar had been withdrawn. A possible explanation, likewise, may be found for subject W's apparently poor response to lactose during period VIII. Although during periods II, III, IV, and V, this child had consistently retained only about one-fifth of his calcium intake (to be exact, 20.7, 21.1, 20.6 and 20.4%, respectively), his per cent retention during the control period unaccountably jumped to 26.6%. If the latter value should represent the maximal extent to which this child could retain calcium, then it is obvious that an effect of lactose could not be demonstrated. However, this child did not respond to lactose during period X, and it is possible that

the value of 10.2% accorded to him for period VIII does represent the maximal effect which lactose would have on his calcium metabolism. On the other hand, the great increase in retention (89%) computed for subject Br may be too high, in view of the fact that his retention of calcium was unduly low during the control period (i.e., it was 16.2% of the intake in contrast to values of 17.4, 19.0 and 17.0, respectively, for periods II, III and IV).

This discussion of the possible factors responsible for the lack of consistency in the data does not alter the fact that each child did show some improvement in his ability to retain calcium during period VIII. It is significant also that these effects were superimposed on any which might have resulted from the administration of liberal quantities of vitamin D. One can conclude, therefore, that the calcium utilization of man, like that of other species, can be influenced by the ingestion of the di-saccharide, lactose.

The metabolism data do not reveal the mechanism by which lactose aids the body in retaining calcium. However, it seems significant that, coincident with the ingestion of lactose there was a statistically significant trend toward a decrease in the calcium lost by way of the kidney. That this decrease in urinary calcium was mainly responsible for the increase in calcium retention is evident when the data for both are computed on the basis of calcium intake; the average percentage decrease in urinary calcium approximates the average percentage increase in calcium retention (subject W, during period X, is the only child who does not show this relationship). However, this is the only study in which, to date, a significant decrease in urinary calcium as a result of lactose feeding has been reported. In fact, Gross ('27) found an increase in the urinary calcium excreted by normal dogs; Outhouse, Smith and Twomey ('38), on the other hand, found no change in the urinary calcium of rats, nor did Robinson, Huffman and Mason ('29) who worked with calves. If subsequent lactose studies on children should confirm these data on pathways of calcium excretion, one might be justified in concluding that lactose exerts its effect, not by rendering the dietary calcium more

absorbable in the intestinal tract, but by making more utilizable that calcium which had found its way into the blood stream. Such a conclusion would seem more tenable if one accepts the contention of Kinsman et al. ('39) that growing children do not excrete endogenous calcium when ingesting inadequate amounts of calcium; if the urinary calcium is not of endogenous origin, it must, then, be unutilized absorbed calcium. It seems logical to believe that the ingestion of lactose, and the possible conversion of a portion of it to an organic acid of a type which could bring about a more complete ionization of the calcium in the blood stream (Greenwald, '38), could make some of this "wasted" calcium available for calcification purposes. On the other hand, the conventional explanation (advanced in 1923 by Dragstedt and Peacock) for the effect of lactose is that calcium absorption from the intestines is increased; in order to fit this theory to the data observed in this study, it would be necessary to assume a decrease in the permeability of the kidney to calcium with a consequent rerouting of this calcium to the intestines where it is excreted.

SUMMARY

The ability of lactose to influence the calcium retention of five little boys ranging in age from 5 to 7 years was investigated. Throughout the 13 to 16 weeks of the experiment, the children were fed a basal dietary to which $CaHPO_4$ was added in order to raise the level of calcium intake to approximately 500 mg. daily; vitamin D also was fed. For 6 to 9 consecutive weeks an additional supplement of lactose (36 gm. daily) was given. Calcium balances were determined, and the increase in percentage retention of calcium during the lactose period was used as the criterion of the effect of lactose on calcium utilization. The values for per cent retention during the non-lactose and lactose periods, respectively, were 26.6 and 29.3 for W, 26.5 and 29.4 for D, 20.1 and 21.5 for C, 16.2 and 30.6 for Br, and 14.0 and 21.1 for Jw. Obviously, the values for the lactose period were higher in every case; they constituted gains over the retentions of the non-lactose period of 10.2, 10.9, 7.0, 88.9 and 50.7% with an average of 33.5.

ACKNOWLEDGMENT

The authors wish to make known their appreciation of the continued cooperation and assistance given by Miss Charlotte Beard and Miss Nellie Ratcliffe, dietitians, and of Mrs. Charlotte Fitzgerald, superintendent of the Cunningham Children's Home where this experiment was conducted.

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THE EFFECT OF PYRAZINE ACIDS AND QUINOLINIC ACID ON THE V-FACTOR[1] CONTENT OF HUMAN BLOOD AND UPON CANINE BLACKTONGUE

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FOUR FIGURES

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In this paper observations are recorded which bear on the question whether nicotinic acid can be replaced in some of its functions by pyrazine monocarboxylic acid, pyrazine 2, 3-dicarboxylic acid, or quinolinic acid. It has been reported that quinolinic acid does not cure canine blacktongue (Woolley et al., '38), and that it does cure pellagra (Vilter and Spies, '39). This distinction is important, if confirmed, as it would be the first demonstration that the nutritional deficiencies underlying the two diseases are not identical, excepting the Chittenden-Underhill syndrome of blacktongue due to vitamin A deficiency (Chittenden and Underhill, '17; Underhill and Mendel, '28; Smith, Persons and Harvey, '37). It has also been reported that pyrazine monocarboxylic acid and pyrazine 2, 3-dicarboxylic acid cure promptly the glossitis of pellagra and that the mono-acid causes an increase in the V-factor content of human blood corpuscles and urine (Bills, McDonald and Spies, '39), as nicotinic acid does (Vilter, Vilter and Spies, '39; Kohn, '38); the pyrazine compounds did not produce in humans the marked vasodilator symptoms which often follow the administration of nicotinic acid.

[1] V-factor = Coenzymes I and II, and possibly unknown related compounds.

EXPERIMENTAL

Quinoxaline was synthesized by the method of Hinsberg (1887) from glyoxal and *o*-phenylene-diamine. Alkaline permanganate oxidation yielded pyrazine 2, 3-dicarboxylic acid (Gabriel and Sonn, '07), M.P. 186° C. Decarboxylation occurred on refluxing the latter for 15 minutes in glacial acetic acid, with an almost theoretical yield of pyrazine monocarboxylic acid, M.P. 221° C. There was no depression of the melting points of these compounds when mixed with samples supplied by Dr. C. E. Bills. Commercial quinolinic acid was used, and in view of the recent report that it may contain a considerable amount of nicotinic acid (Koser et al., '40) our sample was tested by treating an aqueous solution with cyanogen bromide and metol (Bandier and Hald, '39) and analyzing the absorption spectrum of the resulting colored solution. It contained less than 0.03% of nicotinic acid. By the same method the samples of the pyrazine acids were shown to be entirely free from nicotinic acid.

Effect on V-factor of human blood in vitro. It has been established that when defibrinated normal human blood is incubated under sterile conditions with nicotinic acid the V-factor content of the blood is increased (Kohn and Klein, '39). We have conducted similar experiments to find whether quinolinic acid and the pyrazine acids would also lead to the synthesis of V-factor. The data are collected in table 1 and it will be seen that neither quinolinic acid nor the two pyrazine acids stimulated the synthesis of V-factor. There is one low aberrant figure among those for nicotinic acid, and one aberrant high figure for pyrazine 2, 3-dicarboxylic acid, but the error of the method warrants disregard of these. (It has been shown that in comparative readings as used here, one assay in three may be expected to err by $\pm$ 7% and one in twenty by $\pm$ 16%, Kohn et al., '39.)

Synthesis of V-factor by blood in vivo. Healthy adult subjects on normal diets were observed. On the first and third experimental days, venous blood was assayed for V-factor (Kohn and Bernheim, '39) to establish a baseline value for

each individual. Drug administration was begun on the third day, after the blood sample had been drawn. The compounds were dissolved in water, approximately neutralized, and were given by mouth three times a day for 3 days, each dose being stoichiometrically equivalent to 5 mg. of nicotinic acid per kilogram body weight. Additional blood samples for assay were drawn after the fifth and ninth doses.

TABLE 1

Increase of V-factor content of human blood cells during incubation with various compounds for 18 hrs. at 34°C.

COMPOUND	CONCENTRATION (MILLIMOLS PER LITER)	PERCENTAGE INCREASE (EACH FIGURE FROM A SEPARATE TRIAL)
Nicotinic acid	0.40	31
	0.81	43, 49, 13, 30
	2.43	43, 41
Pyrazine mono carboxylic acid	0.81	4, 0
	2.43	6, 5, 0
	7.29	4
Pyrazine di-carboxylic acid	0.40	−2
	0.81	2, 12
	2.43	7
	4.1	3
	12.3	2
Quinolinic acid	0.81	0
	2.43	3, −5

For each datum the result was expressed as the ratio of V-factor concentration after incubation in the experimental tube to the concentration in an otherwise similar tube from which the test compound was omitted. Thus for 0.40 millimolar nicotinic acid the ratio was 1.31, therefore the V-factor concentration had increased 31% over the control tube.

The results are plotted in figure 1, and show that none of the test substances caused a significant rise in the V-factor content of the blood cells. After a lapse of 4 weeks the effect of nicotinic acid was determined on two of the subjects, and a third acted as control without treatment. Four doses of nicotinic acid were given to each subject over a period of 24 hours, each dose being 5 mg. per kilogram body weight. Blood samples drawn before and after the dosing period showed ele-

vation of the blood V-factor of the subjects who received nicotinic acid, comparable with similar elevation reported previously (Kohn, '38).

One of the subjects (P.H.) suffered a severe flushing reaction after taking pyrazine monocarboxylic acid, and another (H.I.K.) experienced "prickly heat" of the skin following one dose of pyrazine 2, 3-dicarboxylic acid.

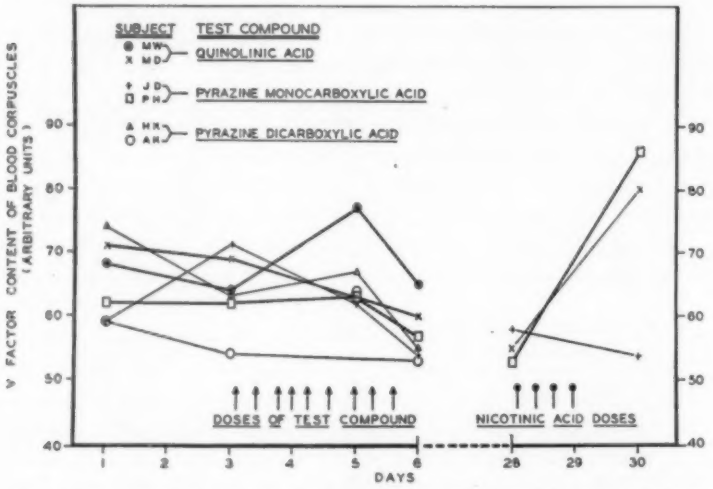

Fig. 1 The effect of oral ingestion of quinolinic acid, pyrazine monocarboxylic acid and pyrazine 2, 3-dicarboxylic acid on the V-factor content of human blood corpuscles. Each dose given was the molecular equivalent of 5 mg. of nicotinic acid per kilogram body weight of the subject. No significant increase occurred with any of the compounds; the increase for MW after treatment on the fifth day may be regarded as within the error of the method as it was not sustained; it compares with the apparent increase for JD before dosing was begun. In the later experimental period nicotinic acid was taken by MD and PH while JD served as control not dosed with nicotinic acid.

Effect in canine blacktongue. Each of the three acids was tested for blacktongue curative activity, by the standardized procedure previously described (Dann and Subbarow, '38), modified by giving each dog 2 mg. riboflavin and 2 mg. thiamin weekly in addition to the basal diet. The results of the curative

tests were variable and uncertain. With each acid, some of the dogs continued to decline after treatment and soon died of blacktongue, while others showed a rapid remission of mouth symptoms, return of appetite, and partial restoration of weight. In the latter animals the appetite generally remained good for some time, but the weight usually began to fall again a few days after treatment ceased, and the mouth signs returned sooner than after a curative treatment with nicotinic acid. No animal was fully cured, as judged by all of the following criteria—amount of weight restored; length of period after treatment until weight declined again; length of periods of restitution of appetite and freedom of mouth signs.

Four dogs were treated with quinolinic acid in doses of 3.33 to 13.75 mg. (In the curative tests, the dose signifies the weight of substance given per kilogram body weight each day for 5 days. Thus a 10-kg. dog on a dose of 3.33 mg. would receive in all 166.5 mg. during the 5-day dosing period. Under our conditions a dose of 2 mg. nicotinic acid causes complete cure.) The best response was seen in dog 21, receiving 13.75 mg.; its weight prior to the dosing period had decreased from 9.3 to 7.7 kg. Following the treatment it increased to 8.5 kg. 2 days after the last dose was given and then began to fall again immediately. The dog was not cured, although it received a dose stoichiometrically five times greater than the dose of nicotinic acid which gives a maximum response.

Seven dogs received pyrazine monocarboxylic acid in doses of 2 to 10.25 mg. None of the animals was completely cured, and there was no regular gradation of increasing response to higher doses. One of the best responses was shown by dog 17, given a dose of 3 mg. in its first attack of blacktongue. Its weight fell from 11.5 to 9.5 kg. before treatment and recovered rapidly to 10.5 and more slowly to 10.7 kg. on the eleventh day after treatment was completed. Then it began to fall rapidly and on the twentieth day after treatment was 8.6 kg. Normal appetite was restored for 20 days and then completely disappeared; the mouth signs returned on the eighteenth day after treatment.

Five dogs received pyrazine 2, 3-dicarboxylic acid in doses of 7.6 mg. to 13.6 mg. (Stoichiometrically 13.6 mg. of the di-acid are equivalent to 10 mg. nicotinic acid.) Only one of these dogs showed any weight restoration at all, although three of them showed some remission of mouth signs. The best response was given by dog 10, receiving 13.6 mg. From a weight of 9.75 kg. it had decreased to 8.8 kg. before dosing commenced, and further declined to 8.15 kg. but rose to 8.75 kg. on the fifth day after the last dose was given and then immediately began to decrease again. Appetite returned to normal but the mouth symptoms never disappeared or showed remission, and 10 days after the last dose treatment with 2 mg. nicotinic acid was begun and gave a complete cure.

These results indicated that none of the three acids tested was as potent a specific in blacktongue as nicotinic acid, although considerable improvement in mouth condition and appetite was produced in some of the dogs. In order to test more rigorously whether any of these compounds can replace nicotinic acid completely if given in sufficient quantity, preventive trials were carried out.

In the preventive tests, dogs were selected which were either still normal after only a few days on the deficient regimen or which showed only slight signs of the onset of blacktongue. A dose of test substance was given subcutaneously each day, until the observation period was terminated. The results obtained on some of the individual dogs are shown in figures 2 to 4, and a summary of the results follows: Five dogs were treated daily with quinolinic acid at levels of from 1 mg. to 20 mg. respectively per kilogram. Those receiving the lower doses declined and were killed for tissue examination when mortally sick with blacktongue. The dog receiving 20 mg. per kilogram was maintained in health, as judged by weight, mouth condition and appearance, for 156 days, after which it was killed (fig. 2, dog 10B). Four dogs were treated daily with pyrazine monocarboxylic acid at levels from 1 to 10 mg. per kilogram; these all declined and were killed for examination. Three dogs were treated with pyrazine 2, 3-dicarboxylic acid

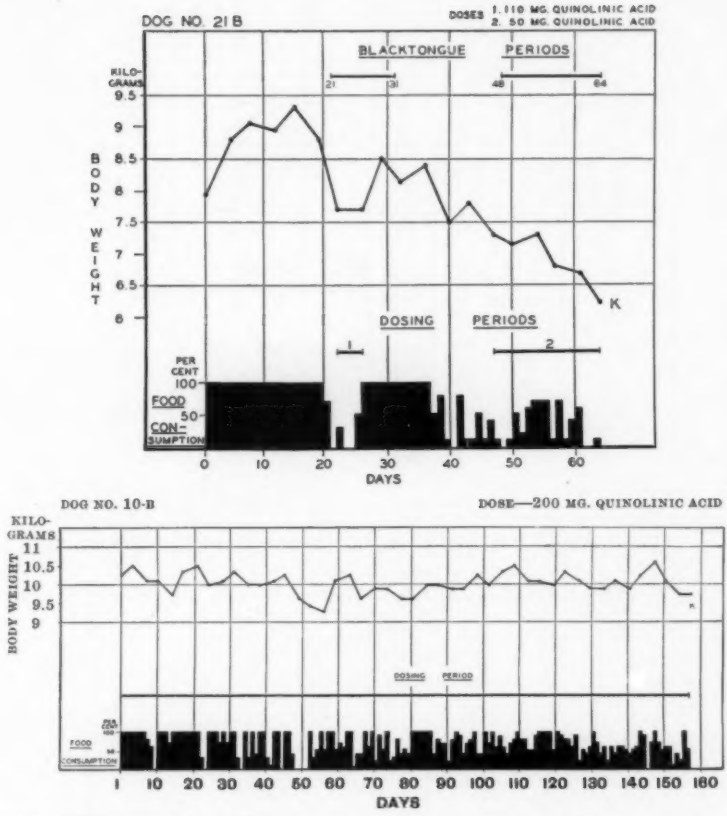

Fig. 2 Effect of quinolinic acid in blacktongue. Dog no. 21B increased in weight for 2 weeks on the basal diet, then developed blacktongue and showed a partial cure after receiving five daily doses each of 11.6 mg. quinolinic acid per kilogram (equivalent to 8.5 mg. nicotinic acid). The dose is calculated on the maximum weight of the dog in health. The mouth signs cleared up, but weight was only partly restored and the appetite returned for less than 10 days. Before the mouth symptoms returned preventive dosing was begun with 50 mg. quinolinic acid (equivalent to 4 mg. nicotinic acid) per kilogram daily. Blacktongue reappeared and the dog declined continuously until sacrificed. Dog 10B was normal at the beginning of the period shown and was given 200 mg. of quinolinic acid daily. Normal health and weight were maintained for 156 days as shown.

Note: "Blacktongue periods" in all figures refer to periods during which the characteristic mouth lesions were present. Food consumption indicates the percentage eaten of a standard weighed portion of the basal diet presented fresh daily to each dog.

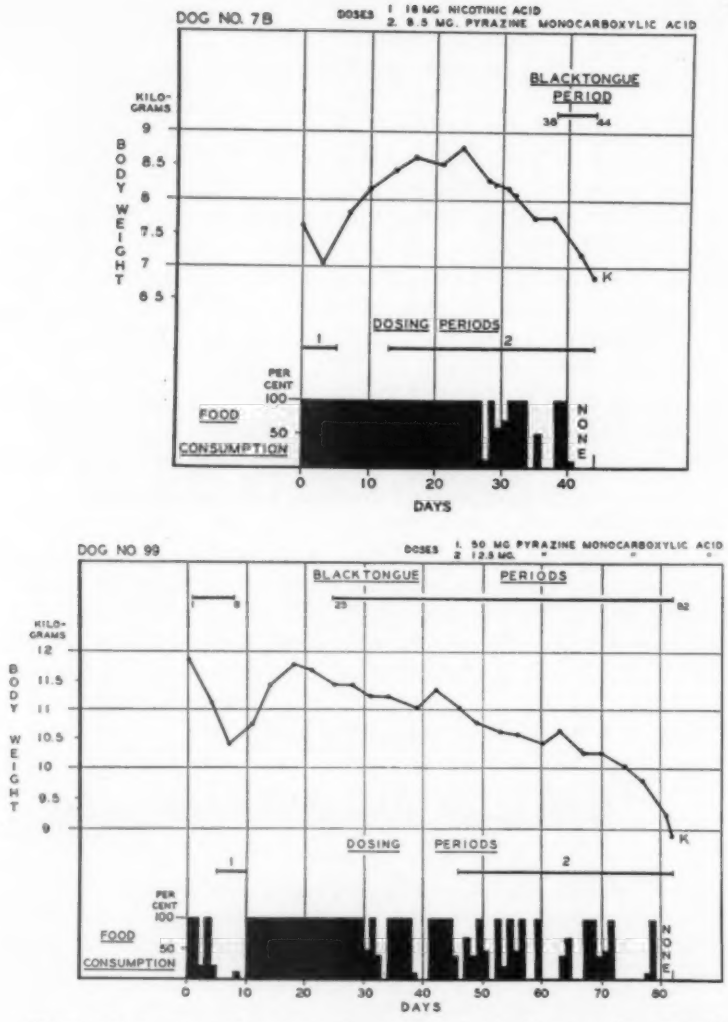

Fig. 3 Effect of pyrazine monocarboxylic acid in blacktongue. Dog 7B did not have blacktongue at the beginning of the period shown but was rapidly declining following an ineffective curative test which restored a normal mouth and appetite but did not prevent continued loss of weight. Five daily doses of 2 mg. nicotinic acid per kilogram caused full recovery. While the dog was quite healthy preventive treatment was begun with 1 mg. pyrazine monocarboxylic acid per kilogram daily, but the animal declined into mortal sickness as rapidly as though no treatment had been given.

Dog 99 first received a standard 5-day curative test and showed recovery for a limited period. The mouth signs soon returned, appetite then decreased, but only a slow loss of weight occurred. Treatment with 1 mg. per kilogram daily did not arrest the decline.

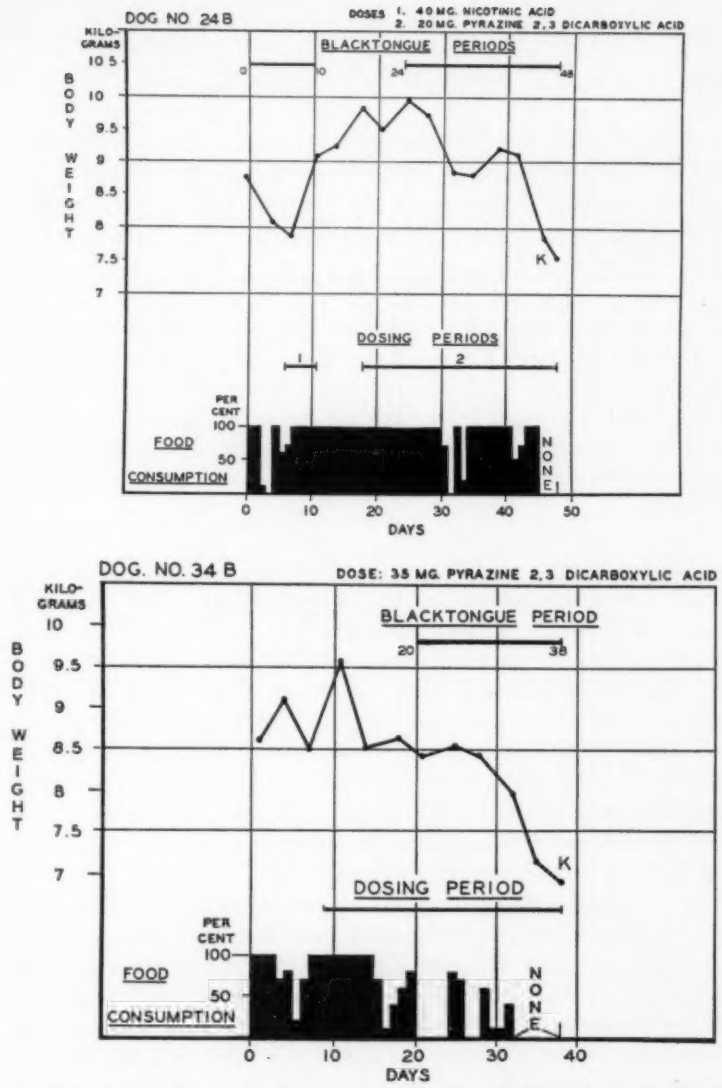

Fig. 4 Effect of pyrazine 2, 3-dicarboxylic acid in blacktongue. At the beginning of the period shown dog 24B was acutely sick following a curative test with an ineffective supplement; it was therefore given the large dose of 4 mg. nicotinic acid per kilogram daily for 5 days. When fully recovered from the attack it was given preventive treatment with 2 mg. pyrazine dicarboxylic acid daily, without retarding the reappearance of blacktongue.

Dog 34B was given the basal diet for the first time and while still healthy on the eighth day preventive treatment was begun with 4 mg. per kilogram daily. This did not give protection from blacktongue.

in doses of from 2 to 4 mg. per kilogram; and these also became sick and were killed when it appeared likely that they could not survive another night. Every dog except 10B showed marked signs of blacktongue before it was killed.

DISCUSSION

Our observations suggest that neither quinolinic acid nor the pyrazine acids raise the blood cell V-factor content when given orally to humans in doses more than twice as great as the amount of nicotinic acid causing at least a 50% increase. Similarly none of the three acids when added to defibrinated blood in vitro caused like nicotinic acid a marked synthesis of V-factor.

The disagreement between our results and those of Vilter and Spies ('39) and Bills et al. ('39) may be due to their use of H. influenzae instead of H. parainfluenzae, used by Lwoff and Lwoff ('37) in their identification of V-factor and employed in all our experiments. Also our subjects were all in normal health whereas they reported observations on pellagrins only; but this seems unlikely to be the cause of the difference as pellagrins and normal humans both show a similar increase in blood cell V-factor following the administration of nicotinic acid (Kohn, '38). A third variation in procedure is in the method of measuring the bacterial growth, which we estimated by photoelectric densitometry.

The experiments with dogs showed that in blacktongue none of the three acids exerted more than a fraction of the activity of nicotinic acid. In none of the curative tests was there a complete recovery although the stoichiometric ratio (largest dose of acid tested ÷ minimum dose of nicotinic acid insuring complete recovery) was 5 for quinolinic acid, and 10 for each of the pyrazine acids.

For an evaluation of the preventive tests it is necessary to adopt a figure for the level of nicotinic acid which will prevent blacktongue and maintain health and weight. From the "standard curves" of Margolis et al. ('38) it is seen that 5 mg. of nicotinic acid per kilogram body weight, spread over 10 days,

will cure a dog of blacktongue and maintain it for 35 days until blacktongue reappears. This gives a figure of $\frac{5}{35} = 0.14$ mg. nicotinic acid daily which is probably somewhat higher than that needed to maintain a dog which is healthy at the beginning of the test period. Again, Birch ('39) has reported that 0.084 mg. per kilogram daily did not afford protection from blacktongue, but 0.13 mg. daily gave protection and permitted slow growth. We shall therefore take 0.15 mg. per kilogram per day as the preventive dose of nicotinic acid. Under our conditions a dose of quinolinic acid stoichiometrically twenty-five times greater was ineffective in preventing blacktongue; but a hundredfold greater dose gave complete protection for 156 days. A sixty-sixfold greater dose of pyrazine monocarboxylic acid and a twentyfold greater dose of pyrazine dicarboxylic acid (in terms of gram-molecular quantities) failed to prevent the appearance of blacktongue.

Our results with quinolinic acid do not conflict with the observation of Woolley et al. ('38) that one dog weighing 5.6 kg. was not cured by a single dose of 150 mg. On the other hand they do conflict with the results obtained on human pellagra by Vilter and Spies. At approximately the same level of dosage Vilter and Spies ('39) found quinolinic acid to be as effective as nicotinic acid (Spies et al., '38). This conflict might indicate a species difference, contrary to our basic assumption that the deficiencies underlying the two diseases are identical. The close correspondence between dietary supplements effective in the treatment of pellagra and blacktongue, both qualitatively (Sebrell, '34) and quantitatively (Ruffin and Smith, '39) argues against this. Since Koser et al. ('40) suggested that commercial quinolinic acid may be contaminated with sufficient nicotinic acid to produce the cures seen by Vilter and Spies, and we now find quinolinic acid much less effective in blacktongue, a re-examination of its activity in pellagra is suggested. It is possible that moderate doses may produce temporary remission of the mouth signs without curing the underlying deficiency, as they do in blacktongue.

The results with the pyrazine acids in blacktongue treatment also conflict with the observations of Bills et al., ('39) on pellagra.

SUMMARY

1. Quinolinic acid, pyrazine monocarboxylic acid or pyrazine 2, 3-dicarboxylic acid taken orally by humans do not cause a rise in blood cell V-factor content.

2. None of the three acids caused a synthesis of V-factor when incubated under sterile conditions with defibrinated human blood.

3. None of the three acids was effective in curative trials on blacktongue. In preventive trials either 5 mg. quinolinic acid per kilogram, 10 mg. pyrazine monocarboxylic acid per kilogram or 4 mg. pyrazine dicarboxylic acid per kilogram daily was insufficient to prevent blacktongue, but 20 mg. quinolinic acid daily was preventive. These figures contrast with 0.15 mg. nicotinic acid daily, which is sufficient to prevent blacktongue.

Our thanks are due to the John and Mary R. Markle Foundation for a grant in aid of this study; to Merck and Company for supplying the thiamin and riboflavin used; and to Dr. C. E. Bills of Mead Johnson and Company for supplying part of the pyrazine acids tested.

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THE LENGTH OF TIME REQUIRED FOR DEPLETION OF VITAMIN A RESERVES IN RANGE CATTLE [1]

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(Received for publication July 17, 1940)

Although considerable information is available as to the time required for depletion of vitamin A reserves in laboratory animals and scattered items may be found for some other species, nutritional literature contains very limited information pertaining to the depletion time of different species under varied conditions. The data presented herein pertain to range cattle over a period of 5 years and are a part of the results of an investigation designed to determine the carotene requirement for fattening beef cattle.

REVIEW OF LITERATURE

The use of cottonseed and its by-products, cottonseed meal and cottonseed hulls, as cattle feeds in the southern states has provided a fertile field for study of vitamin A deficiency in the bovine. In many cases, cattle fattened on cottonseed meal and cottonseed hulls became dull, listless, went "off feed," scoured and died as a result of the ration. Prior to 1900, cattle feeders knew this abnormal result as "fat sick-

[1] This report is part of an investigation to determine the carotene requirement for fattening beef cattle which became cooperative with the United States Department of Agriculture, Bureau of Animal Industry in 1935. A part of the data in this paper were presented in a paper entitled "Carotene Requirements for Fattening Beef Cattle" by J. H. Jones, J. K. Riggs, G. S. Fraps. J. M. Jones, H. Schmidt, R. E. Dickson, Paul E. Howe and W. H. Black, which was published in the Proceedings of the American Society of Animal Production, 1938, pages 94–102.

ness'' and recognized that it was often accompanied by inflammation of the eye or total loss of sight.

Connell and Carson (1896) attempted to produce ''fat sickness'' experimentally in native 3- and 4-year-old steers by feeding cottonseed meal and cottonseed hulls, but were unable to do so in periods of 180 days. This was the first attempt to produce the condition experimentally, and the admitted failure of these workers to do so may probably be explained through the fact that the experimental animals were aged steers from the area near the Gulf Coast of Texas where the annual rainfall approximates 40 inches and the growing season is about 250 days in length. The importance of these factors will be brought out later in this paper.

The involvement of the eye in this condition was thus early recognized, and the work of Halverson and Sherwood ('30) later substantiated it. Guilbert and Hart ('35) and Dickson, Jones and Schmidt ('35) established the relationship of night blindness and vitamin A deficiency in cattle. Halverson and Sherwood ('30) and Dickson, Jones and Schmidt ('35) have shown conclusively that the major factor in so-called cottonseed meal poisoning in cattle is the vitamin A deficiency of rations which have commonly been compounded from cottonseed meal and hulls to the exclusion of other feeds.

Halverson and Sherwood reported the appearance of unusual conditions of eyes, eye lesions, and dullness of sight without lesions, in a large percentage of steers after 88 days of feeding in dry lot on a ration composed exclusively of cottonseed meal and hulls. No mention was made of feeding prior to the time the test was begun. They also found that dairy heifers about 1 year of age may be fed such a ration for 150 to 250 days before acute symptoms of deficiency occur.

Guilbert and Hart ('34) observed night blindness in two Shorthorn steers, 11 and 17 months of age at the beginning of the experiment, in 248 and 252 days, respectively, on a vitamin A deficient ration after removal from conditions which afforded excellent opportunity for storage of vitamin A.

Mead and Regan ('31) reported development of avitaminosis A in calves 1 to 3 months after the diet was changed from whole milk and grain to a concentrate mixture low in vitamin A. Likewise, Moore ('39) found that two calves raised to 90 days of age on whole milk and grain and then fed skim milk together with a low carotene grain ration became night blind at 128 and 153 days of age or after 38 and 63 days, respectively, on the deficient ration. Bechdel and Skaggs ('36) observed symptoms of vitamin A deficiency in twelve dairy calves approximately 6 months of age after 70 days of feeding on a ration containing 37.2% of cottonseed meal but no roughage. Bechdel and Ward ('36) produced symptoms of avitaminosis A in five of six calves ranging in age from birth to 8 weeks by feeding a concentrate mixture of cereal grains and oil meals with poor quality timothy hay for 11 to 24 weeks.

These data indicate not only the variability in onset of symptoms of deficiency in different individuals but also the effect of age and the importance of feed supply and its vitamin A potency prior to the time the animals are placed on vitamin A deficient diets. The purpose of this paper is to present data pertaining primarily to the effect of age and seasonal range conditions upon the length of time required for depletion of vitamin A reserves in feeder cattle and calves as ordinarily marketed from ranges in the western part of Texas. It should be of especial value because of the large number of animals used in the work.

EXPERIMENTAL

The general plan of these experiments was to deplete the animals of vitamin A reserves in the body by feeding them a ration containing practically no carotene until they became night blind and then adding to the ration definite quantities of carotene in alfalfa meal during fattening periods varying in length. This paper is concerned only with the time required for depletion; therefore the discussion will be limited to that phase of the work.

Criteria for determining depletion

Previous investigations with cattle at this station (Dickson, Jones and Schmidt, '35) as well as at the California Station (Guilbert and Hart, '35) have shown that night blindness is the first symptom of vitamin A deficiency ordinarily detectable. The night blind condition appeared to be of progressive nature in nearly all cases in this investigation and therefore was measured in terms of degrees as shown below:

0—normal, no indication of defective night vision;
1—cautious, but can see objects at least 5 feet away;
2—cautious, but can see objects at a distance of 2 feet to a few inches;
3—totally night blind, cannot see.

Two consecutive observations of first degree night blindness or other definite assurance of affected night vision or of vitamin A deficiency were considered evidence of depletion. The animals were tested for night blindness out-of-doors by driving them about the pens and through 8-foot alleys formed by two board fences in which were located various obstacles such as inverted V-type hog troughs and movable panel barriers of unpainted wood or painted white on one side and black on the other. Testing was done at night as soon as twilight had passed.

Diets used and method of feeding

The depletion diets fed the various groups of cattle are shown in table 1. Cottonseed hulls constituted the roughage for all groups except 6 and 8 which received sorghum fodder having a carotene content of approximately 4 μg. per gram. Group 7 also received sorghum fodder, which contained 2 parts per million of carotene, for the first 58 days and was then changed to cottonseed hulls. The grain fed to groups 5, 6, 7 and 8 consisted of ground milo heads ranging in carotene content from 0.3 to 0.5 of 1 part per million. The total quantities of carotene derived from these feeds undoubtedly supplied a considerable part of the daily needs of the animals thereby lengthening somewhat the time required for depletion as com-

pared to what it would have been had cottonseed hulls formed the sole roughage in every case; however, the data were not materially affected. All of the animals were fed twice daily as groups except those in group 5 which were individually fed.

TABLE 1

Percentage composition of depletion diets

Components of ration	GROUP NUMBER							
	1	2	3	4	5	6	7	8
Tankage		3	3	3	3			
Cottonseed meal	12	10	10	10	15	13	12.5	15
Sorghum grain					30	38	25.0	28
Sorghum fodder [1]						49		57
Cottonseed hulls	88	87	87	87	50		62.5	
Limestone flour [2]	None				1	Ad lib	Ad lib	Ad lib
Salt	Ad lib	Ad lib	Ad lib	Ad lib	1	Ad lib	Ad lib	Ad lib

[1] Group 7 was fed sorghum fodder for the first 58 days and cottonseed hulls thereafter.

[2] Groups 3 and 4 were fed limestone flour at the rate of 0.10 pound per head daily.

Animals used

Two hundred seventy head of cattle have been used during the 5 years of this study. They were grade Hereford steers and heifers taken directly from the S.M.S. Ranch in Dickens County, Texas, to dry lots at Substation no. 7, Spur. In each case they were taken directly off the range at the time of purchase. The calves as groups ranged in age from 3 to 8 months and in weight from 225 to 467 pounds. The yearlings [2] were approximately 16 months old and averaged 559 pounds in weight. Table 2 shows the time required for depletion and gives the description of each group.

DISCUSSION

Wide variations are evident in the time required for depletion of different individuals (table 2). This appears to be true particularly in the case of older animals. The time re-

[2] Animals under 10 months of age are usually considered calves; 10 to 18 months, yearlings; and 18 to 30 months, 2-year-olds.

TABLE 2

The length of time required for depletion of vitamin A reserves in cattle of various ages

	GROUP NUMBER							
	1	2	3	4	5	6	7	8
Year	1935–1936	1936–1937	1937–1938	1938–1939	1939–1940	1938–1939	1939–1940	1939
Animals used: Class	*Steer yearlings*	*Steer calves*	*Steer calves*	*Steer calves*	*Heifer calves*	*Steer calves*	*Steer calves*	*Steer calves*
Number	48	40	50	50	30	10	20	12
Age in months	16	6–8	6–8	6–8	4–6	4–6	3–5	3–5
Length of depletion period in days:								
45– 59	..	..	..	..	..	..	..	6
60– 74	..	..	..	..	..	..	11	6
75– 89	..	..	..	10	1	10	6	..
90–108	..	9	1	20	25	..	2	..
109–128	5	1	16	15	4	..	1	..
129–148	9	18	19	3	..	..	..	..
149–168	10	8	13	2	..	..	..	..
169–188	5	2	0	0	..	..	..	..
189–208	5	2	1	..	..	..	..	..
209–228	13	..	..	..	..	..	..	..
229–248	0	..	..	..	..	..	..	..
249–268	1	..	..	..	..	..	..	..
Average	178 ± 37	136 ± 25	138 ± 21	107 ± 21	98 ± 7	86 ± 5	79 ± 17	56 ± 6
Range	128–266	101–206	96–194	82–155	82–112	83–90	65–131	46–61

quired for the yearlings in 1935–1936 ranged from 128 to 266 days with an average of 178 and a standard deviation of 37. One steer remained on the deficient ration for 381 days without becoming completely night blind, but showed numerous other symptoms of deficiency including swelling of the joints. This suggests a possible difference between individuals in ability to utilize carotene or vitamin A for a given physiological function. In contrast, the widest variation found in calves used in the years 1936 to 1939 was from 101 to 206 days. These wide variations in time required to become night blind show that different individuals have different reserves of vitamin A in the body. The smaller degree of variability in the younger animals also tends to indicate smaller storage in their case.

In the work with fattening cattle, the time required for depletion is determined by the body reserves of vitamin A which are affected by (1) the age of the animals, and (2) the vitamin A potency of the range forage. The effect of age may readily be seen in table 2. Younger animals have smaller reserves because of a shorter period of feeding on grass.

Appreciable variations are evident in the length of time required to deplete calves of the same age classification from year to year. The condition of the grass or range is determined by the amount and distribution of rainfall. Fraps, Copeland and Treichler ('34) have found that the feed of cows must be high in vitamin A potency in order for the animals to continue to produce butterfat high in vitamin A potency. Silage and ordinary hays and fodders apparently will not supply enough vitamin A potency to maintain a high content of vitamin A in butterfat. Green growing pasture grasses appear to be needed to maintain the production of butterfat high in vitamin A. When the leaves of plants dry, become brown and die, the vitamin A value may be completely lost (Coward, '25). In regions of frequently occurring drouths this suggests a relationship between rainfall and the vitamin A potency of the range forage.

Although the total annual precipitation for the years 1935 to 1938 varied only 4.51 inches and the departure from the 28-year average of 21.03 inches was not great, there was considerable difference in the rainfall for the 4 months, July, August, September and October, just preceding the time when all of these cattle except group 8 were removed to dry lot. During the fall of 1935, 1936 and 1937 the range grass made a good growth and was green until frost, but in 1938 as well as in 1939, there was little rainfall after the middle of July, and the grass was mostly dry from that time until frost. The average time required for depletion of the calves was 136 days in 1936–1937 and 138 days in 1937–1938. In these 2 years the July–October rainfall was relatively high. In 1938, however, when the July–October precipitation totalled only 4.93 inches, as compared to 15.50 and 12.26 inches in the 2 previous years, the average time required for calves of the same age was only 107 days. Slightly younger animals, taken from the range in the fall of 1939 when the July–October precipitation was 6.15 inches, required an average of 98 and 79 days for depletion of the two respective groups. One hundred per cent of these animals were depleted at the end of 128 days.

All of the groups shown in table 2 except no. 8 were purchased in the fall. After those in group 6 became depleted in an average of 86 days, the question arose as to the time for depletion required for animals of the same age which had been on the range not only during the fall but also during the dry winter months. The twelve animals in group 8, obtained for follow-up investigation, were removed from the range on March 17th just before the grass began spring growth and were started immediately on a depletion ration. At the end of 61 days they were all night blind, the average time being 56 days and the range 46 to 61 days.

Forty heifer calves approximately 5 months old were taken from the range on October 25, 1939, and placed on a vitamin A deficient ration composed of tankage, cottonseed meal, ground

milo heads and cottonseed hulls. They were divided into two groups of thirty head and ten head, respectively. The group of thirty, shown in table 2, required an average of 98 days for depletion. In addition to the depletion ration the group of ten head was fed 1000 μg. of carotene per 100 pounds live weight daily in dehydrated alfalfa leaf meal from the time they were brought in. These calves required an average of 113 days for depletion or only 15 days longer than those which were fed the unsupplemented depletion ration.

These findings indicate that range animals may go for considerable periods in the feed lot without suffering from vitamin A deficiency, but it is evident that these periods are not long enough for fattening to a high degree which may require 200 to 240 days in the case of young animals. This must naturally be modified according to the degree of deficiency of the fattening rations and the conditions which prevailed on the range where the cattle grazed.

SUMMARY AND CONCLUSIONS

A table showing the depletion time for 260 range cattle ranging in age from 3 to 16 months is presented. The data bear out the statement of Bessey and Wolbach ('39) that the accumulation of vitamin A in the body tends to increase with age and is dependent on the character of the diet. In dry years when only limited amounts of green vegetation are available on the range the time required for vitamin A deficiency to occur is less than in years of more abundant rainfall. This fact as well as the age of the animals must be taken into consideration when cattle are fed in dry lot for considerable periods of time on rations which do not supply an abundance of carotene or vitamin A.

One thousand micrograms of carotene (fed in dehydrated alfalfa leaf meal) per 100 pounds live weight daily added to the depletion ration for calves lengthened the average time required for the occurrence of night blindness only 15 days.

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PLACENTAL AND MAMMARY TRANSFER OF VITAMIN E IN THE RAT [1]

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In a previous report (Mason and Bryan, '38) evidence was presented indicating that placental transfer of vitamin E in the rat is negligible, and that mammary transfer is decidedly limited. When suckling rats, denied access to any source of vitamin E other than that obtained through the placenta and mammary glands of mothers receiving approximately three times the minimal daily requirement of the vitamin, were subsequently reared on an E-deficient diet, testicular degeneration appeared in the males within 30 to 40 days after weaning (51 to 61 days of age); while female offspring, inseminated at an average age of 66 days, consistently resorbed during their first gestation period. Increasing the maternal intake of vitamin E to fifteen and to 400 times the minimal, prior to the tenth day of lactation, induced a delay of approximately 20 and 30 days, respectively, in the onset of testis changes but failed to prevent first pregnancy resorptions in the female offspring. Other observations, involving exchange of litters between mothers given excess vitamin E and those on the stock diet, indicated that the vitamin E storage responsible for the delayed sterility in male offspring was largely, if not entirely, due to mammary transfer of the vitamin.

[1] This investigation was aided by a grant to Vanderbilt University School of Medicine from the Division of Medical Sciences of the Rockefeller Foundation.

The studies reported in the present paper represent an attempt to increase still further the state of E-hypervitaminosis in the pregnant and lactating female rat, to discriminate more fully between placental and mammary transfer of vitamin E under such conditions, and to measure the extent of this transfer by direct and indirect methods.

EXPERIMENTAL

The procedure used was essentially the same as that adopted in the previous study (Mason and Bryan, '38), except for the increased maternal intake of vitamin E. Normal female rats were mated and immediately transferred to the high-E diet, containing 20% by weight of a concentrate prepared from wheat germ oil by molecular distillation.[2] The composition of this diet, and that of our vitamin E deficient diet, was as follows:

High-E diet		*E-deficient diet*	
Casein (unextracted)	22%	Casein (unextracted)	20%
Cornstarch	54	Corn starch	48
Vitamin E concentrate	20	Lard	18
Salts	4	Salts	4
Brewers' yeast	1 gm. daily	Brewers' yeast	8
Cod liver oil	6 dr. daily	Cod liver oil	2

By the use of non-scatter food cups and daily records of food consumption, the intake of the concentrate was measured and the number of mean fertility doses for adult female rats estimated on the basis of bio-assays simultaneously made on this material. The limited supply of the latter permitted the use of only four rats at this exceedingly high level of vitamin E intake. At the fourteenth day of the 21-day period of lactation the high-E diet was replaced by the E-deficient diet. It was estimated that the daily vitamin E intake of each rat during pregnancy and during the first 2 weeks of lactation, respectively, was at least 2000 and 3000 times the minimal daily requirement for female rats; the larger intake during lactation

[2] We are indebted to the Research Laboratories of General Mills, Inc., Minneapolis, Minn., for generous supplies of this concentrate.

being due to the increased food consumption characteristic of this period.

Differentiation between placental and mammary transfer was accomplished by exchange of litters simultaneously delivered by high-E rats and by stock rats of the breeding colony, such exchange being made immediately after parturition and before any lactation occurred. The lactating mothers in both groups were transferred to the E-deficient diet on the fourteenth day of lactation in order to prevent the suckling young from obtaining any additional vitamin E through voluntary consumption of the maternal diet. Thus, offspring born of high-E mothers and fostered by stock rats represented the placental-transfer group; those born of stock rats and suckled by high-E rats constituted the mammary-transfer group. Six males delivered and suckled by the same high-E mother, representing the combined effect of placental and mammary transfer, were so indistinguishable from those of the mammary-transfer group in subsequent responses that they have been included with the latter in the summarized data (table 1). Special precautions were taken to prevent cutaneous absorption of the vitamin by the suckling young through physical contact with the high-E diet. The twenty female and twenty-six male offspring were weaned at the twenty-first day and continued on the E-deficient diet.

RESULTS

Extent of placental and mammary transfer of vitamin E. The left testis and epididymis of each male rat were removed by operation after different periods of experimental feeding and at a time when the early stages of testicular degeneration were expected. The latter were frequently ascertained by examination of stained smears obtained by puncture of the testis with a small hypodermic needle prior to operation. The other testis and epididymis were removed at autopsy 5 to 20 days later. After a careful examination of representative histological sections from both organs the testes were classified according to the predominant stage, or stages, of degeneration

present, using the arbitrary divisions of the degenerative process described by Mason ('26). On the basis of the histopathology of the germinal epithelium, supplemented by observations on the relative number and distribution of sperm and sloughed germ cells in the ducts of the epididymis, it was possible to estimate within very close limits the time at which the earliest morphological changes made their appearance. The latter analysis was aided by an extensive series of observations which we have made to determine the usual time interval between different stages of the degenerative process which, in general, is completed within a period of 15 to 20 days in our standardized male rats.

The time of onset of testis injury in rats of the placental-transfer group was not significantly different from that in the standardized controls (table 1).[3] However, when compared with the latter, the rats of the mammary-transfer group exhibited an average delay of 47 days in the appearance of demonstrable testis injury, which may be compared to the delay of about 20 and 30 days previously observed (Mason and Bryan, '38) when the maternal intake of vitamin E was approximately fifteen and 400 times the minimal, respectively. It thus appears that a maternal intake of vitamin E approximately 2000 to 3000 times the minimal permitted no demonstrable transfer of vitamin E through the placenta but increased the mammary transfer considerably over that occurring at much lower levels of vitamin intake, when delay in onset of testis degeneration was used as a measure of vitamin E storage in the newly weaned offspring.

[3] In earlier studies (Mason and Bryan, '38) it was noted that testicular degeneration appeared within 30 to 40 days after weaning in offspring of breeding females given access to both the stock and E-deficient diets during the first 10 days of lactation and to the deficient diet alone thereafter. In order to avoid the late-weaning paralysis which appeared in about one-fifth of litters prepared in this manner, we have since routinely transferred mothers and their litters from the stock diet to the deficient diet at the fourteenth day of lactation. The progeny, continued on the E-deficient diet after weaning on the twenty-first day, are referred to as our "standardized" rats. These standardized males show testis injury within 35 to 50 days after weaning (average, 40 days).

The female offspring in each group were bred to normal males. Successful matings occurred within an average of 40 days after weaning, at which time testicular injury was appearing in males of the control and placental-transfer groups. Since the results of previous studies of a similar type indicated that complete resorption would occur in all instances, several rats in each group were given doses of a vitamin E

TABLE 1

Showing extent of vitamin E storage, due to placental and mammary transfer, in young rats reared from weaning upon an E-deficient diet

GROUP	DELIVERED BY MOTHER FED	NURSED BY MOTHER FED	MALES				FEMALES			
			Number of rats	Days on experiment at onset of testis degeneration [1]			Vitamin E conc. fed on 4th day of pregnancy	Number of rats	Responses	
				Min.	Max.	Aver.			Neg.	Pos.[2]
							mg.			
S (standardized controls)	Stock diet	Stock diet [3]	42	34	50	40	0	35	35	0
							10	15	15	0
							15	9	9	0
							20	31	15	16
P (placental transfer)	High-E diet	Stock diet [3]	11	38	47	42	0	6	6	0
							10	2	2	0
							20	3	2	1
M (mammary transfer)	Stock diet	High E-diet [3]	15	72	98	87	0	4	4	0
							10	3	0	3
							20	2	0	2

[1] Estimated on the basis of histological examination of testes and epididymides removed at operation and at autopsy after different periods on the E-deficient diet.

[2] Based on the presence of two or more living fetuses at autopsy on the sixteenth day of pregnancy.

[3] Replaced by vitamin E-deficient diet during last week of lactation.

concentrate representing (1) a subminimal dose of 10 mg. consistently giving negative results in bio-assay tests, and (2) a dose of 20 mg. giving a positive response in approximately 50% of tests (see group S; table 1). All rats were autopsied on the sixteenth day of pregnancy and the presence of two or more viable fetuses was taken as a criterion of positive response. The advantages of this method of evaluating responses

will be discussed in a later report. The data are summarized in table 1.

The untreated rats of all three groups resorbed during their first pregnancy. Rats of the placental-transfer group responded to the vitamin E dosage in essentially the same manner as did the controls, while those of the mammary-transfer group were definitely more sensitive. This indicates that they had received through the mammary secretions of the foster mother an appreciable amount of vitamin E which, however, was insufficient to permit completion of their first gestation. Although these deductions are based upon a very limited number of animals, they are in full accord with observations made on littermate brothers (table 1) and with the results of other experiments discussed below.

Direct measurement of placental transfer. Female rats maintained on the stock diet relatively low in vitamin E, and others maintained on a diet of fresh raw wheat germ (supplemented with inorganic salts) for about 1 month prior to as well as during the period of gestation, were allowed to deliver on a coarse wire screen in order to permit the young to drop out of reach of the mother before they had an opportunity to suckle. In some instances, the rats were autopsied on the twenty-first day of pregnancy so as to obtain placentae and uteri as well as the fetuses for bio-assay tests. These tissues, and the newborn offspring, were frozen at 0°C. and fed, within about 10 days, to standardized females during early stages of their first pregnancy (table 2). As much as 200 gm. of newborn from stock rats gave only a subminimal response, characterized by the presence of a single well developed, but dead, fetus at the sixteenth day of pregnancy. On the basis of our experience with assay tests of this type it is estimated that approximately 250 gm. would have been required to give positive responses equivalent to those given by 50 gm. of newborn (ten young) delivered by high-E mothers. It is apparent that a demonstrable but definitely limited amount of vitamin E can pass the placental barrier. The low concentration of the vitamin in individual offspring of high-E mothers,

amounting to one-tenth the prophylactic dose for adult females, explains our failure to detect an appreciable storage of E in the placental-transfer group of rats (table 1) by the indirect method of approach. Other bio-assay tests of placentae and uteri obtained from high-E rats autopsied just prior to, or immediately after, delivery (table 2) indicate that the concentration of vitamin E on the maternal side of the placental barrier was more than five times that present in the fetuses or newborn.

Indirect measurement of mammary transfer. In order to determine the storage of vitamin E which must be present at weaning to permit a delay in the onset of testis changes comparable to that observed in the mammary-transfer experiment, two groups of twenty-one and seven standardized male rats were fed, during the first 3 days after weaning, varying doses of two different concentrates of wheat germ oil representing approximately 1, 2, 4, 8, 12 and 16 times the mean fertility dose.[4] These preparations were administered as ether solutions evaporated on a casein-dextrin mixture and fed as single doses after a preliminary starvation period of 6 to 10 hours. The onset of testicular injury was determined according to the procedure outlined in an earlier paragraph. From the data presented in table 3, it will be noted that the amount of concentrate X required to effect a delay in onset of testis injury

[4] The term "mean fertility dose," first proposed by Bacharach ('38), denotes the dose capable of giving a positive response in 50% of assay tests. It must be kept in mind that the response given at critical levels of dosage may be appreciably modified by differences in the period over which the test dose is administered. At the beginning of these studies we were feeding the total dose as a single dose on the fourth day of the first pregnancy, fed on a portion of the diet. For sake of uniformity this procedure was followed in frequent assays of the concentrates during the 10-month period of their use in the studies reported, although we feel that direct oral administration of divided doses over 5 or 10 days is much to be preferred. In estimating the mean fertility doses fed, it has been necessary to allow for a slight decrease observed in the potency of the concentrates during the course of these experiments. A few differences exist between the figures presented here, in relation to the doses given and the delay of testis injury observed, and those cited in a preliminary report (Mason, '39). The latter estimates were based upon a combined evaluation of the two groups of males in table 3 and were made before the complete bio-assay and histological data had been obtained and analyzed.

TABLE 2

Bio-assay tests showing the vitamin E storage in the newborn offspring of rats fed the low-E stock diet, and in the newborn, suckling young (24 hours old), placenta, uterus and mammary gland of rats maintained on a diet of fresh raw wheat germ for 1 to 2 months

TISSUE FED		UTERUS AT 16TH DAY L. D. R.[1]	TISSUE FED		UTERUS AT 16TH DAY L. D. R.[1]	TISSUE FED		UTERUS AT 16TH DAY L. D. R.[1]	TISSUE FED		UTERUS AT 16TH DAY L. D. R.[1]
Amount in grams	Days of pregnancy fed		Amount in grams	Days of pregnancy fed		Amount in grams	Days of pregnancy fed		Amount in grams	Days of pregnancy fed	
Newborn of stock rats			Newborn of high-E rats			Newborn of high-E rats (suckled first 24 hours)			Mammary gland (at term)		
200	3–7	0–1– 7	50	4–6	5–1– 2 (4)	30	4–6	9–0–0 (8)	10	5–6	11–0– 0 (10)
120	4–7	0–0–13	50	4–6	2[2]–1– 6 (0)	20	4–6	10–0–0 (10)	5	5–6	12–0– 0 (11)
100	4–7	0–0– 8	40	4–6	0–0– 9	20	4–6	10–0–1 (10)	3.5	5–6	1[2]–2– 4 (0)
90	4–8	0–0– 8	40	4–6	0–0–11	15	5–6	12–0–0 (12)	2	5–6	0–0–11
60	3–5	0–0–10	40	4–6	0–0– 9	15	4–6	3[2]–3–5 (1)	2	5–6	0–0– 4
50	3–6	0–0– 5	20	4–7	0–0– 4	Placenta (21-day preg.)			(24 to 48 hours after delivery)		
50	3–5	0–0– 8	20	4–8	0–0– 8	20	5–7	7–0–0 (7)	7.7	5–6	8–0– 0 (8)
40	3–8	0–0–10	20	3–5	0–0– 6	10	5–6	2[2]–3–3 (0)	3.5	5–6	9–0– 0 (9)
30	2–5	0–0–10	15	3–5	0–0– 5	6.5	5–6	0–0–6			
			15	5	0–0–10	Uterus (21- to 22-day preg.)					
			15	5	0–0– 9	10	5–6	8–0–2 (8)			
			15	5	0–0–11	2.2	5–6	0–0–9			

[1] Laparotomy was performed on all rats at the sixteenth day of pregnancy, and the number of live fetuses, dead fetuses and resorption sites recorded. The figures in parentheses indicate the number of fetuses delivered at term.

[2] Some uncertainty existed concerning the viability of fetuses recorded as living at laparotomy.

TABLE 3

Showing effect of single doses of vitamin E concentrates when fed to male and female E-deficient rats immediately after weaning

CONCENTRATE		TESTICULAR DEGENERATION IN MALES						REPRODUCTION IN FEMALES	
		Left testis (operation)		Right testis (autopsy)		Days on experiment at onset of degeneration (estimated) aver.		Days on experiment at time positive mating	Outcome of first pregnancy
Amount fed, mg.	Number of mean fertility doses	Days on experiment	Stage of degeneration	Days on experiment	Stage of degeneration				
Concentrate X [1]									
0	Controls	45	1	60	3–5	42	40.5	35	Resorption
		45	1–2	60	5	41		38	Resorption
		45	1–2	60	5	41		41	Resorption
		45	2–4	60	5	38		42	Resorption
		45	N–1	60	4–5	43		42	Resorption
10	1	45	1	60	4–5	42	46.7	43	Resorption
		60	1–2	75	5	55		50	Resorption
20	2	45	N	60	N	...	67.2	31	Resorption
		60	N	75	N–1	73		37	Resorption
		60	N–1	75	4–5	58		39	Resorption
		75	1–2	90	4–5	71		45	Resorption
		75	3–4	90	5	67		..	
40	4	75	N	90	1–2	86	87.6	34	Resorption
		90	N	105	1	102		37	Resorption
		90	1	105	3–5	86		41	Resorption
		90	2–4	105	5	83		48	Resorption
		90	3–4	105	5	81		..	
80	8	90	N	105	N	...		36	Resorption
		90	N	110	5	95		44	Resorption
120	12	90	N	120	3–5	108		49	Resorption
160	16	120	1	135	4–5	116		..	
Concentrate Y [1]									
0	Controls	50	2–3	70	5	43	44.5	29	Resorption
		60	4–5	80	5	46		30	Resorption
		...	...	...	..	...		37	Resorption
100	4	50	N	75	2–4	68	67	29	Resorption
		60	N	70	1–2	66		43 [2]	Resorption
		...	..	...	...	...		48 [2]	Resorption
200	8	60	N	80	N–1	77	76	27 [2]	Resorption
		80	2	90	4–5	75		36	Resorption
		...	.. .	...	...	...		36	Resorption
		...	..	...	...	...		49 [2]	Resorption
		...	..	...	...	...		50 [2]	Resorption
300	12	...	..	...	...	...		30	Resorption
		...	..	...	...	...		33	Resorption
400	16	...	..	...	...	...		31	Resorption
		...	..	...	...	...		33	Resorption

[1] Concentrate X was an extract prepared from wheat germ oil, furnished us through the kindness of Dr. H. A. Mattill of the Department of Chemistry, State University of Iowa, Iowa City. Concentrate Y was the same molecular distillate of wheat germ oil as used in the high-E diet.

[2] Rats which failed to give a positive response when fed one-half the mean fertility dose of concentrate Y on the fourth day of their first pregnancy.

comparable to that observed in rats of the mammary-transfer group (table 1) corresponded to four times the mean fertility dose. In other words, the individual rats of the latter group must have acquired during lactation an average storage of E equivalent to four mean fertility doses. This, in turn, represents a storage forty times greater per rat than that resulting from placental transfer. However, on the basis of weights of 5 gm. and 40 gm. for newborn and newly weaned rats, respectively, the actual concentration of vitamin E per gram of tissue would be in a ratio of 1 to 5.

We have calculated that the rats of the mammary-transfer group possessed a storage of vitamin E equivalent to about one five-hundredth of the total intake of the mother during lactation. Since the four high-E rats suckled an average of 6.25 progeny, it is apparent that the average litter received through the mammary secretions about one-eightieth of the maternal intake during lactation. It must be stated, however, that the maternal storage depots may have been fully saturated by the high intake of E during pregnancy. Furthermore, since we have no data on vitamin excretion in the high-E rats, one cannot state what proportion of the vitamin intake was actually absorbed and retained by the maternal tissues. In terms of mean fertility doses, concentrate Y proved decidedly less effective in male rats than did concentrate X, but the number of animals tested was too limited to warrant very definite conclusions.

In contrast to the clear-cut response given by males to vitamin E dosage at the end of weaning, thirty-three littermate sisters treated in like manner and mated after an average experimental period of 39 days consistently resorbed during their first pregnancy (table 3). In fact, five of these rats failed to give positive responses when fed subminimal doses of one of these concentrates on the fourth day of pregnancy. These observations first suggested that the young female rat absorbs and stores vitamin E less effectively than the male, or else dissipates an excess of E more rapidly during prepubertal growth.

In order to obtain more specific information on this question thirty-eight standardized females were fed (according to the method used in the preceding study) either three or six times the mean fertility dose of concentrate Y, administered in three consecutive daily doses at periods of 14 to 30 days after weaning. Since single doses of this same size, administered in the same manner but on the fourth day of pregnancy, had been shown to give positive responses in standardized females, it was felt that the possibilities of partial destruction or poor absorption of the vitamin would thus be

TABLE 4

Showing the effectiveness of six times (120 mg.) and three times (60 mg.) the mean fertility dose of vitamin E concentrate (Y) fed as single doses to standardized female rats at intervals prior to the time of positive mating

120 MG. DOSE				60 MG. DOSE			
Days fed prior to mating	Implantation sites at autopsy on the 16th day of pregnancy			Days fed prior to mating	Implantation sites at autopsy on the 16th day of pregnancy		
	L. D. R.[1]	L. D. R.	L. D. R.		L. D. R.	L. D. R.	L. D. R.
27–24	0–0–12	1–0– 8	0–0–11	15	1–1– 7		
23–20	0–0–11	0–0–11	9–0– 0	14	0–0–10	5–2–4	
18	0–0– 9	2–2– 7	4–1– 2	13	0–0–10	1–0–9	3–0–8
17	5–2– 4	5–2– 3		11	2–0– 8	6–0–5	
15	2–0– 1	3–0– 6	10–0– 0	10	8–1– 3	9–0–1	
13	3–0– 5	4–1– 3		9	2–0–10	2–5–3	
12	3–0– 6	5–0– 6	10–0– 0	8	11–0– 0	8–1–2	
10	6–0– 4	8–0– 2		5	2–0– 8	7–0–3	8–0–1

[1] L (live fetuses), D (dead fetuses) and R (resorption sites).

eliminated. By subsequent regulation of the time of mating in the dosed females it was possible to obtain a series in which the second day of dosage occurred at intervals of 5 to 27 days prior to the time of insemination. The data obtained (table 4) indicate that six and three times the mean fertility dose became ineffective when fed about 3 and 2 weeks, respectively, prior to the day of mating. These results suggest that the females used much of the vitamin E administered for metabolic activities other than those related to reproductive functions. This is in accord with the observation that 2 mg. oral doses of

this same concentrate, after losing two-fifths of its potency during a year's storage, represented close to the minimal daily requirement for preventing sterility in standardized rats of both sexes (Mason, '40).

Apparently the male and female rats utilize minute daily supplements of vitamin E with equal efficiency in the satisfaction of their reproductive needs, and there is reason to believe that their capacity to absorb and store vitamin E is essentially the same. The seemingly paradoxical nature of the response of the two sexes to vitamin E received through the mammary secretions (table 1) or administered shortly after weaning (table 3) can now be satisfactorily explained on the basis of certain qualitative differences in sex requirements for vitamin E. These differences are characterized by the fact that vitamin E becomes effective in males prior to the twenty-fifth day after weaning, if irreversible disturbances in the seminiferous epithelium are to be prevented, whereas the reproductive needs of the female rat become acute only after conception and implantation and, consequently, arise at any stage of the reproductive period (Mason, '40).

Direct measurement of mammary transfer. Bio-assay data presented in table 2 indicate that vitamin E storage in the progeny of high-E mothers increases more than threefold during the first 48 hours of lactation [5] and that the mammary gland tissue itself represents a concentrated source of vitamin E. In fact, no other tissue from rats maintained for a similar period on the raw wheat germ diet has shown as high a vitamin E potency (unpublished studies). These findings are in accord with observations relative to the extent, and indirect measurement, of mammary transfer discussed above. Measurement of mammary transfer by direct bio-assay of the entire carcasses of newly weaned rats was not attempted.

[5] No attempt was made to discriminate between the vitamin E actually stored by the tissues, and that in the undigested milk present in the stomach, of the suckling young.

DISCUSSION

In view of the indispensability of vitamin E for the normal growth and development of the fetus in the rat, it is surprising that the maximum storage which the latter acquires during intrauterine life is so limited. It must be kept in mind that the observations presented in this report have dealt primarily with the extent of vitamin E storage in newborn and newly weaned rats as a result of placental or mammary transfer of the vitamin, irrespective of that which may have been directly utilized for general metabolic processes in the developing fetus and in the suckling rat. This metabolic utilization, which cannot be measured experimentally, is probably not very great in view of what is known regarding the minimal daily requirements of rats during the post-weaning period (Mason, '40).

Studies relating to the distribution of carotene and of vitamin A in the human placenta as well as in the fetal tissues point to an exceedingly limited transfer of these substances through the placenta (Wendt, '36; Gaehtgens, '37, '37 a). Likewise, the limited transfer observed in rats and rabbits can be augmented but little by feeding large amounts of vitamin A during pregnancy (Dann, '32, '34). The same appears to be true of the placental transfer of vitamin D in the rat (McCollum et al., '27). The decidedly restricted transfer of vitamin E across the placental barrier might be construed as a design of nature to protect the fetus against an excess of this vitamin, or to conserve for use of the maternal organism any vitamin over and above that required by the fetus. However, vitamins A and D behave in like manner and there is no evidence that an excess of any of the fat-soluble vitamins is really deleterious to the fetus. Consequently, the limitations of placental transfer of vitamin E must be attributed to the physiological selectivity of the placental barrier which, insofar as the fat-soluble vitamins is concerned, may represent a function of their molecular size.

Our observations concerning the more effective, but still definitely limited, transfer of vitamin E through the mammary

gland is in close accord with what is known regarding the mammary transmission of vitamin A in the rat and in man (Dann, '32, '36) and of vitamin D in rats (McCollum et al., '27). Additional studies are necessary to determine whether the concentration of vitamin E is greater on one side of the mammary barrier than on the other.

The earlier observations of Evans and Burr ('27), and unpublished studies from this laboratory in which 20% of butter fat from cows on green pasture was required in diets otherwise deficient in vitamin E in order to confer fertility on rats reared on the diet from weaning, indicate that mammary transfer of vitamin E in cattle is very limited. Advantage has recently been taken of the low content of vitamin E in cows' milk for the production in dogs of a condition of muscular dystrophy attributed to an inadequacy of vitamin E (Anderson et al., '39). A satisfactory approach to the problem of experimental vitamin E deficiency in various mammalian forms which do not thrive for long periods on the purified and concentrated type of diet customarily used for rats and mice might be facilitated by rigid restriction of the maternal intake of vitamin E prior to the time of weaning, followed by the use of diets composed largely of milk from animals receiving a low intake of E.

The time of onset of testis degeneration has proved a very sensitive index of the extent of initial storage of vitamin E in newly weaned rats, and constitutes a useful criterion of the rapidity and degree of vitamin E depletion in any colony of rats being used for experimental E-deficiency. In addition to observations presented in this report, one of us (K.E.M.) has had an opportunity to test this criterion in another laboratory possessing a large colony of rats devoted to the bio-assay of vitamin E.[6] For the purposes of this test, one or more male rats were included in litter groups of newly weaned

[6] We are greatly indebted to Mr. A. L. Bacharach, Director of the Biochemical Division of the Glaxo Laboratories, Ltd., Greenford, Middlesex, England, for his kindness in placing the facilities of his laboratory at our disposal and for his generous cooperation in these studies.

females being placed on the E-deficient diet. Smears of the testis and epididymis were obtained from males autopsied at intervals after the sixtieth day of age. Differences of about 20 days in the onset of testis injury were readily detected by this method in two series of E-deficient rats which differed only in their vitamin E intake during lactation. No great experience is required to ascertain the onset of early stages of the degenerative process, characterized particularly by the appearance of numerous abnormal spermatozoa scattered individually or in clumps in different portions of the smear, without the necessity of examining histological sections of these organs.

In the early reports of Evans and Burr ('27) the condition of "initial fertility" observed in their E-deficient female rats, and noted by many other investigators since that time, was explained on the assumption that young animals are born with appreciable stores of vitamin E which, although slowly dissipated by the various metabolic processes during early growth and development, are supplemented by traces of E present in the purified diet to such an extent that a variable number of females are enabled to complete one or more gestations before sterility intervenes. The observations reported here, and elsewhere (Mason and Bryan, '38; Mason, '40), indicate that (1) the maximum result of combined placental and mammary transfer of vitamin E falls far short of conferring fertility on female rats deprived of any other source of the vitamin, (2) the usual components of E-deficient diets do not contain sufficient traces of E to augment appreciably the animal's stores of the vitamin, (3) the storage which newly weaned rats acquire through voluntary consumption of stock diets of variable E-content represents the major cause of "initial fertility" in rats reared on E-deficient diets.

SUMMARY AND CONCLUSIONS

1. Comparisons have been made between the placental and mammary transfer of vitamin E by stock rats receiving an adequate but low intake of this vitamin, and by rats given

approximately 2000 to 3000 times the minimal daily requirement of vitamin E throughout pregnancy and the first 2 weeks of lactation.

2. When measured by the delay in onset of testis degeneration in male offspring otherwise deprived of vitamin E, no placental transfer was demonstrable but mammary transfer was sufficient to cause an average delay of 47 days in the appearance of testicular injury. This may be compared with a delay of about 30 and 20 days previously observed when the maternal intake of E was approximately 400 and fifteen times the minimal, respectively. Indirectly measured, the maximum mammary-transfer storage in individual rats amounted to about four times the mean fertility dose for adult females.

3. Placental and mammary transfer of vitamin E to female offspring otherwise deprived of vitamin E failed to prevent resorptions during their first pregnancy. These somewhat paradoxical observations are discussed in relation to qualitative time differences in the vitamin E needs of the two sexes.

4. On the basis of direct bio-assay tests the newborn from high-E rats (fed fresh raw wheat germ for 1 to 2 months) contained approximately five times as much vitamin E as those from the stock rats, indicating a demonstrable but decidedly limited transfer of the vitamin across the placenta to the fetus amounting to about one-tenth the mean fertility dose in each newborn rat. The concentration of vitamin E in the placenta and uterus of high-E mothers was about five times greater than in the full term fetus or newborn, but considerably less than in the mammary gland. After 24 hours of lactation the vitamin content of the newborn increased more than threefold.

5. It is concluded that placental transmission of vitamin E in the rat is exceedingly limited, and that the vitamin is much more readily transferred through the mammary gland to the milk. These findings are discussed in relation to the experimental production of vitamin E deficiency and to the question of placental and mammary transmission of other fat-soluble vitamins.

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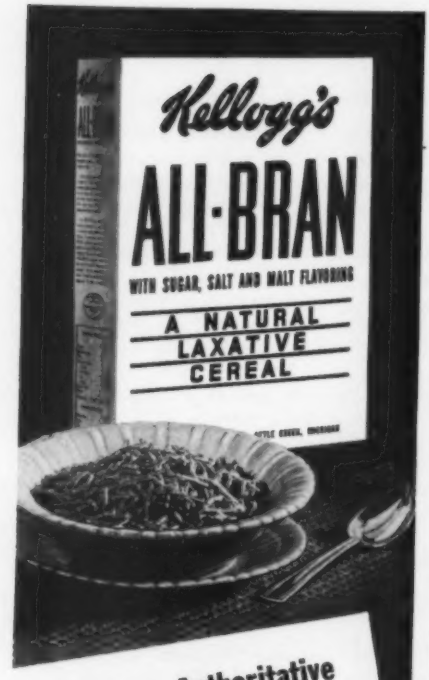

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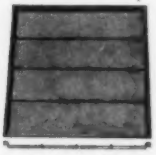

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